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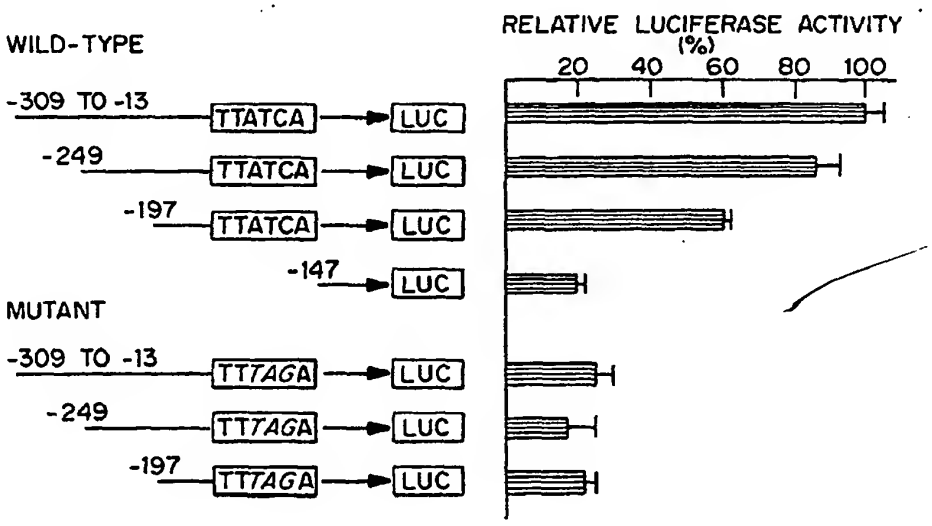

(57) Abstract

DNA molecules and methods for the regulated expression of a gene in endothelial cells or megakaryocytes, are described, wherein the 5' flanking region of the P-selectin gene, or portions thereof, is ligated to the 5' end of a gene. The DNA molecules are also used as probes for screening individuals with abnormal levels of expression of P-selectin, or for production of pharmaceutical compositions to inhibit inflammation by inhibition of expression of P-selectin. These DNA molecules can also be used to identify and isolate previously unknown proteins which are involved in regulation of gene expression.

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EXPRESSION CONTROL SEQUENCES OF THE P-SELECTIN GENE

Background of th Invention

This invention is generally in the field of compositions and methods for the treatment and prevention of inflammatory responses involving P-selectin (formerly, GMP-140 or PADGEM) binding reactions, particularly adhesive interactions between platelets, leukocytes, and endothelial cells.

The adherence of leukocytes to vascular surfaces is a critical component of the inflammatory response, and is part of a complex series of reactions involving the simultaneous and interrelated activation of the complement, coagulation, and immune systems. Leukocyte adherence to vascular endothelium is a key initial step in migration of leukocytes to tissues in response to microbial invasion. The initial rolling contacts of leukocytes with the endothelium are mediated by the selectins, a family of receptors that interact with cell-surface carbohydrate ligands (reviewed in McEver, *Curr. Opin. Cell Biol.*, *4*, pages 840-859 (1992); Lasky, *Science*, *258*, pages 964-969, (1992)). These transient adhesive interactions allow time for leukocytes to become activated by signaling molecules that are released from the endothelium or the underlying tissues. Upon activation, leukocytes functionally upregulate members of the integrin family of adhesion receptors. The integrins strengthen adhesion by binding to immunoglobulin-like counter-receptors on the endothelial cell (McEver, *Curr. Opin Cell Biol.*, *4*, pages 840-859 (1992)). Adhesion and signaling molecules function cooperatively to regulate leukocyte recruitment during the inflammatory response.

Leukocytes also adhere to activated platelets, through interactions of P-selectin on the activated platelet surface with carbohydrate ligands on the leukocyte surface (McEver, in Structure, Function, and Regulation of Molecules Involved in Leukocyte Adhesion, pages 135-150 (Lipsky et al., eds., Springer-Verlag, New York, 1993)). Platelet-leukocyte interactions may serve as important links between the hemostatic and inflammatory responses to tissue injury.

The coagulation and inflammatory pathways are regulated in a coordinated fashion in response to tissue damage. For example, in addition to becoming adhesive for leukocytes, activated endothelial cells express tissue factor on the cell surface and decrease their surface expression of thrombomodulin, leading to a net facilitation of coagulation reactions on the cell surface. In some cases, a single receptor can be involved in both inflammatory and coagulation processes. For example, the Mac-1 receptor on leukocytes, a member of the CD11-CD18 group, mediates phagocytosis and serves as a receptor for the degradation product of complement C3bi, is involved in one pathway of adherence of leukocytes to endothelium, mediates granulocyte aggregation, and binds coagulation Factor X.

Proteins involved in the hemostatic and inflammatory pathways are of interest for diagnostic purposes and treatment of human disorders. An example is P-selectin, formerly known as GMP-140 (granule membrane protein 140) or PADGEM, an integral membrane glycoprotein with an apparent molecular weight of 140,000 as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). P-selectin contains an N-terminal lectin-like domain, followed by an

epidermal growth factor-like module, a series of consensus repeats related to those in complement-binding proteins, a transmembrane domain, and a cytoplasmic tail, as described in the parent application, United States Serial No. 07/320,408, filed March 8, 1989, the teachings of which are incorporated herein. P-selectin is a member of the selectin family of adhesion receptors that mediate leukocyte interactions with vascular endothelium or platelets (McEver, *Curr. Opin. Cell Biol.*, *4*, pages 840-849 (1992); Lasky, *Science*, *258*, pages 964-969 (1992); Bevilacqua and Nelson, *J. Clin. Invest.*, *91*, pages 379-387 (1993)). The human P-selectin gene spans over 50 kilobases (kb) and contains 17 exons, most of which encode structurally distinct domains (Johnston et al., *J. Biol. Chem.*, *265*, pages 21381-21385 (1990)).

P-selectin was first purified (as GMP-140) from human platelets by McEver and Martin, *J. Biol. Chem.*, *259*, pages 9799-9804 (1984). Monoclonal and polyclonal antibodies to P-selectin were also prepared, as reported by McEver and Martin (1984) and P.E. Stenberg, et al., *J. Cell Biol.*, *101*, pages 80-886 (1985). The protein is present in alpha granules of resting platelets but is rapidly redistributed to the plasma membrane following platelet activation, as reported by Stenberg, et al., (1985). The presence of P-selectin in endothelial cells and its biosynthesis by these cells was reported by McEver, et al., *Blood*, *70(5)* Suppl. 1:355a, Abstract No. 1274 (1987). In endothelial cells, P-selectin is found in storage granules known as the Weibel-Palade bodies. When platelet or endothelial cells are activated by mediators such as thrombin, the membranes of the storage granules fuse with the plasma membrane, the soluble contents of the granules are released to

the external environment, and membrane bound P-selectin is presented within seconds on the cell surface, where it mediates adhesion of neutrophils, monocytes, and subsets of lymphocytes (McEver, in Structure, Function, and Regulation of Molecules Involved in Leukocyte Adhesion, pages 135-150 (Lipsky et al., eds., Springer-Verlag, New York, 1993).

The expression of P-selectin, as observed by immunohistochemistry (McEver et al., *J. Clin. Invest.*, *84*, pages 92-99 (1989)) and Northern blot analysis (Johnston et al., *Cell*, *56*, pages 1033-1044 (1989)), is restricted to megakaryocytes and endothelial cells. Under certain circumstances, steady-state levels of mRNA and protein are increased by inflammatory mediators such as tumor necrosis factor and endotoxin (Sanders et al., *Blood*, *80*, pages 795-800 (1992); Weller et al., *J. Biol. Chem.*, *267*, pages 15176-15183 (1992); Hahne et al., *J. Cell Biol.*, *121*, pages 655-664 (1993)). Thus, an understanding of the molecular mechanisms that control transcription of the P-selectin gene may help clarify the mechanisms for gene expression in megakaryocytes and endothelial cells and for regulation of leukocyte adhesion in response to tissue injury.

The promoters of several genes whose expression is restricted to endothelial cells or megakaryocytes have been partially characterized (Lee et al., *J. Biol. Chem.*, *265*, pages 10446-10450 (1990); Wilson et al., *Mol. Cell Biol.*, *10*, pages 4854-4862 (1990); Ravid et al., *Mol. Cell Biol.*, *11*, pages 6116-6127 (1991); Romeo et al., *Nature*, *344*, pages 447-449 (1990); Uzan et al., *J. Biol. Chem.*, *266*, pages 8932-8939 (1991)). The GATA element, initially recognized in erythroid-specific promoters, plays an important role in expression of

some of these genes (Wilson et al., (1990), Romeo et al., (1990)). However, this element is not sufficient to mediate tissue-specific expression, as expression of the GATA-binding proteins does not directly correlate with expression of the genes containing GATA elements (Yamamoto et al., *Genes & Dev.*, *4*, pages 1650-1662 (1990)). A functional ETS element has been identified in the megakaryocyte-specific gene for glycoprotein IIb (Prandini et al., *J. Biol. Chem.*, *267*, pages 10370-10374 (1992); Lemarchandel et al., *Mol. Cell. Biol.*, *13*, pages 668-676 (1993)), but this element is also found in genes expressed in other tissues (see, for example, Macleod et al., *Trends Biochem. Sci.*, *17*, pages 251-256 (1992)).

It is an object of the present invention to characterize and provide DNA and RNA sequences of the 5′ flanking region of the human P-selectin gene and to provide methods of using these sequences to specifically express P-selectin and other genes in endothelial cells and megakaryocytes.

It is another object of this invention to provide nucleic acid probes for screening for individuals with abnormal levels of P-selectin.

It is a further object of this invention to provide compositions and methods including the DNA or RNA sequences of the 5′ flanking region of the human P-selectin gene for inhibiting or regulating P-selectin expression to control the inflammatory and hemostatic processes involving endothelial or megakaryocytic cells.

Summary of the Invention

The 5′ flanking region of the P-selectin gene contains the regulatory sequences necessary for expression of P-selectin in endothelial and megakaryocytic cells. These regulatory sequences

are demonstrated to be useful to specifically express other genes in endothelial and megakaryotic cells, both *in vitro*, for example, in tissue culture, and *in vivo*, for example, in transgenic animals. In addition, these regulatory sequences can be used as probes to screen for individuals with abnormal levels of P-selectin and to make pharmaceutical compositions for the regulation or inhibition of P-selection expression in individuals having or predisposed to inflammation. Furthermore, sequences of the 5′ flanking region of the P-selectin gene can be used to identify and isolate previously unknown proteins which are involved in regulation of gene expression.

Brief Description of the Drawings

Figure 1 is a schematic of the nucleotide (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequence of endothelial cell P-selectin that was determined from a composite of four overlapping cDNAs: lambda GMPE1-lambda GMPE4. The relative positions of each of the cDNAs are shown by the solid arrows. The dotted arrows indicate regions found in some clones, but deleted in others. The numbering of the nucleotide sequence was arbitrarily started at the first base following the adapter oligonucleotide sequence of the most 5′ clone. The translated amino acid sequence of the open reading frame is given in the single-letter code. The initiating methionine was assigned to the first in-frame ATG sequence that agreed with the consensus sequence for initiation of translation. The stop codon is shown by the asterisk. The thin underlines show the matching positions of amino acid sequences determined from the N-terminus and from 26 peptides of platelet P-selectin. The signal peptide corresponds to

positions -41 to -1. The putative transmembrane domain is heavily underlined. The cysteine residues are circled and potential asparagine-linked glycosylation sites (NXS/T) are shown by the dark circles. Two potential polyadenylation signals in the 3′ untranslated region are underlined and overlined.

Figure 2 is the structure of the 5′ flanking region of the P-selectin gene. The map shows the cloned region (scale in kb) with the positions of exons (numbered 1-3) and the bacteriophage and plasmid clones used. The region of the 5′ flanking region sequenced is shown by the open box. All restriction sites for the following enzymes are shown on the map: B = *Bam*HI, E = *Eco*RI, Ev = *Eco*RV, H = *Hind*III, and X = *Xba*I.

Figure 3 illustrates the strategy to identify transcriptional start sites of the P-selectin gene and the primers used for primer extension studies and anchored polymerase chain reaction (PCR) and the probes used for RNase protection assays.

Figure 4 shows a summary of transcriptional start sites in the 5′ flanking region (Seq ID No:5) of the P-selectin gene. Vertical arrowheads, vertical arrows, and horizontal arrows indicate, respectively, the positions of the transcriptional start sites determined by primer extension, RNase protection and anchored-PCR cloning. Each horizontal arrow represents an independent cDNA clone obtained by anchored PCR with sequence beginning at the indicated position between nucleotides (nt) 4768 and 4842 of SEQ ID NO. 5.

Figure 5 demonstrates transient expression analysis of P-selectin gene promoter activity. On the left are diagrams of the P-selectin promoter-luciferase fusion constructs. Vertical bars indicate the restriction sites used to make the

constructs. The PCR primers used to generate the shorter constructs are depicted with short arrows. The constructs are aligned with the 5′ P-selectin gene sequences numbered relative to the translational start site. Constructs p1339RLUC and p701RLUC have 5′ flanking sequence oriented in the reverse direction. On the right is plotted the relative luciferase activity of bovine aortic endothelial cells (BAEC) transfected with each construct. Activity is graphed as a percentage, with 100% equal to 24,000 light units per 25 μg of cellular protein. The data represent the means ± SD of at least three independent experiments. Duplicate transfections were performed in each experiment; the variation between duplicates did not exceed 10% of the mean for the experiment.

Figure 6 demonstrates cell-specific expression of the 5′ flanking sequence of the human P-selectin gene. The P-selectin-expressing BAEC and the P-selectin-nonexpressing cell lines HeLa, 293, and COS-7 were transfected with the indicated constructs. Parallel transfections with the positive control plasmid pRSVLUC were simultaneously performed. Luciferase activity is expressed as light units per 25 μg of cellular protein. The data represent the means ± SD of three independent experiments. Duplication transfections were performed in each experiment.

Figure 7 is the mutational analysis of the GATA element. Three wild-type truncated chimeric constructs, and their three corresponding mutant constructs in which the TTATCA sequence was changed to TTTAGA, are depicted on the left of the figure. These constructs were transfected into BAEC, and the luciferase activities were measured as percent relative luciferase activity. The data represent the means ± SD of three independent experiments.

Duplicate transfections were performed in each experiment; the variation betw en duplicates did not exceed 5% of the mean for the experiment.

Figure 8 demonstrates the binding of nuclear proteins to the regulatory sequence encompassing the GATA element. The sequences of the upper strand oligonucleotides were used as probes and competitors. The P-selectin GATA and the nonconsensus GATA are from the indicated regions of the 5′ flanking sequence of the human P-selectin gene. The mutant oligonucleotide contains three nucleotide changes in the core GATA motif of the wild-type P-selectin sequence; the same changes were used in the mutant expression constructs listed in Figure 7. The endothelin-1 GATA oligonucleotide corresponds to the functional GATA element in the human endothelin-1 promoter (Wilson et al., *Mol. Cell. Biol.*, *10*, pages 4854-4862 (1990)).

Figure 9 shows five different recombinant gene constructs used to make transgenic mice. Each construct contains a different portion of the 5′ flanking region of the P-selectin gene fused to the *lacZ* gene. Arrows indicate the orientation of the portion of the 5′ flanking region of the P-selectin gene fused to *lacZ*. Construct 2 is like Construct 1, except that the portion of the 5′ flanking region of the P-selectin gene has been fused in the opposite orientation so that P-selectin gene promoter function is directed away from *lacZ*.

Detailed Description of th Invention

I. Isolation, cloning and charact rization of th gene encoding P-selectin and regulatory sequences associated with the P-selectin gene.

As used herein, unless otherwise specified, the term nucleic acid refers to DNA and the equivalent RNA.

Cloning of the gene for P-selectin was first reported by G.I. Johnston, R.G. Cook and R.P. McEver in Abstract 1238 Supplement II *Circulation, 78(4)* (October 1988). Oligonucleotides were prepared based on N-terminal amino acid sequencing of P-selectin peptides and used to screen a human endothelial cell cDNA library. A 3.0 Kilobase (kb) clone was isolated which encoded a protein of 727 amino acids. An N-terminal domain of 158 residues containing many cysteines, lysines, and tyrosines, is followed by an EGF-type repeating domain structure, and eight tandem repeats of 62 amino acids each, except for the sixth tandem repeat which has 70 amino acids. The repeats are homologous to those found in a family of proteins that include proteins regulating C3b and C4b, but are unique in having six conserved cysteines per repeat instead of the typical four. These are followed by a 24 amino acid transmembrane region and a 35 amino acid cytoplasmic tail.

As reported by Johnston, et al., in Abstract 1218, *Blood* Suppl. *72*, 327a (November 1988), the gene for P-selectin has been localized to the long arm of chromosome 1, where genes for a number of C3b/C4b regulating proteins have been mapped.

As also reported, there appears to be at least two forms of the protein derived from alternative splicing of mRNA: a soluble form and a membrane or granule bound form. Both forms generally have a 186 bp insertion, encoding a ninth 62 amino acid

tandem repeat, between the sixth and seventh tandem repeats of the sequence. The soluble protein has a deletion that removes 40 amino acids including the transmembrane region.

The conclusion that P-selectin serves as a receptor for the adherence of leukocytes to activated endothelial cells and platelets was based on several observations. First, the rapid appearance of P-selectin on the surface of endothelium stimulated with thrombin or histamine parallels the inducible adherence of neutrophils to endothelium stimulated with these agonists. In addition, platelets interact with neutrophils or monocytes only after activation with agonists such as thrombin which cause redistribution of P-selectin to the cell surface; platelet agonists such as ADP which do not induce degranulation and surface expression of P-selectin do not cause platelets to adhere to leukocytes. Second, in endothelium, P-selectin is concentrated in postcapillary venules, where E-selectin is concentrated. The concentration of both proteins in this region is important because postcapillary venules are the predominant sites for binding of leukocytes to platelets prior to their migration across the endothelium into the tissues. Third, purified P-selectin coated on tissue culture microtiter wells mediates specific adherence of purified neutrophils to the wells. Fourth, polyclonal and monoclonal antibodies to P-selectin block 60-90% of the adherence of neutrophils to cultured human umbilical vein endothelial cells stimulated with histamine. Fifth, the cDNA-derived amino acid sequence of P-selectin indicates that its structure is remarkably similar to that of E-selectin, an endothelial cell protein known to bind

neutrophils. Sixth, leukocytes adhere to cells transfected with P-selectin cDNA.

The following methods were used for the production and characterization of P-selectin, antibodies thereto, and nucleotide sequences encoding P-selectin.

Protein Sequencing.

P-selectin was isolated and purified from human platelet membranes. On one occasion it was reduced under an atmosphere of nitrogen by the addition of dithiothreitol (20 mM, final concentration) and alkylated in the presence of iodoacetamide (Bray, et al., *Proc. Natl. Acad. Sci. USA, 83*, pages 1480-1484 (1986). It was then digested with trypsin. The resultant peptides were isolated by two-step, reverse-phase, high performance liquid chromatography (HPLC) using previously described methods (Rosa, et al., *Blood, 72*:593-600 (1988). On a second occasion, P-selectin was reduced and alkylated in the presence of 50 μCi [^{14}C]-iodoacetamide (Amersham) before unlabeled iodoacetamide was added. It was then gel-purified (Bray, et al., 1986) and electroeluted into 25 mM Tris, 192 mM glycine, pH 8.0, containing 0.1% Triton X-100 (Jacobs and Clad, *Anal. Biochem., 154*, pages 583-589, 1986). One milligram of P-selectin was digested with endoglycosidase Glu-C (Boehringer-Mannheim Biochemicals) at a ratio of 1:10 $^{w}/_{w}$ at 37°C. After 6 hr, an equal amount of protease was added and the mixture was incubated for a further 14 hr at 37°C. Peptides were isolated by reverse-phase HPLC as described by Rosa, et al., (1988), except that the initial separation on the C4 HPLC column was carried out using ammonium acetate buffer and the second separation on the C18 HPLC column was carried out using trifluoroacetic acid. Fractions containing

the purified peptides were concentrated to 50 μl and kept frozen before sequencing. Amino acid sequences were determined from the N-terminus of the intact protein and from the peptides by using a gas-phase protein sequencer (Applied Biosystems Model 470A) (Rosa, et al., 1988). Cysteine residues were identified by their elution profile on the HPLC system used by the protein sequencer, and confirmed by measuring ^{14}C radioactivity in duplicate aliquots from each sequencing cycle.

cDNA Screening.

Based upon a portion of the amino acid sequence data, two pools of a 35-mer oligonucleotide probe, designed according to codon usage tables (Lathe, *J. Mol. Biol.*, *183*:1-12 (1985), were synthesized. The complementary strand was used to allow hybridization to RNA by Northern blotting. Inosine was used in one position because the third base of a glycine codon showed no preferential nucleotide. The sequences of the pools were: POOL 1 (SEQ ID NO. 1):

```
5'-GC TGT CCA CTG ICC GAG GTT GTC ACA GCG CAC AAT-3'
                      A               A
                      C               T
                                      C
```

POOL 2 (SEQ ID NO. 2):

```
5'-GC TGT CCA CTG ICC GAG GTT GTC ACA TCT CAC AAT-3'
                      A               C
                      C
```

On Northern blots, both oligonucleotide probes hybridized to a 3.6 kb transcript from poly(A)$^+$ RNA isolated from CHRF-288 cells, a leukemia cell line with megakaryocyte-like properties (Witte, et al., *Cancer*, *58*:238-244 (1986). Pool 2 probes hybridized more strongly and were therefore used to screen a cDNA library by standard procedures (Maniatis, et al., Molecular Cloning: A Laboratory

Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1982). Approximately 1.4 million recombinant phage from an unamplified human endothelial cell lambda gt11 library (Ye, et al., *J. Biol. Chem.*, *262*, pages 3718-3725 (1987) were plated out on *E. coli* Y1088 at a density of 200,000 plaques per 230-mm square plate of NZCYM agar. Duplicate nylon filters (Hybond-N, Amersham) were lifted, denatured, neutralized, and incubated at 42°C for 24 hr in prehybridization solution containing 5x standard saline citrate (SSC) [1x SSC is 150 mM NaCl, 15 mM sodium citrate], 5x Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA], 0.2% SDS, and 200 μg/ml herring sperm DNA. The 35-mer oligonucleotide probe 2 was end-labeled with [gamma-^{32}P]ATP by 5'-polynucleotide kinase and was added to prehybridization solution to give a specific activity of 1 x 10^6 cpm/ml. The filters were hybridized at 42°C overnight and the final washing conditions were 2x SSC, 0.1% SDS at 52°C. Positive plaques, identified by autoradiography of filters, were rescreened twice using the same probe and purified. The positive cDNA inserts were isolated from an agarose gel following digestion of phage DNA with *Sal*I or *Eco*RI. Either enzyme could be used because, during the library construction, cDNAs were ligated to adapter oligonucleotides containing a *Sal*I restriction site as well as an *Eco*RI site (Ye, et al., 1987). The inserts were subcloned into pIBI20 (IBI Biotechnologies, Inc.) for restriction mapping and DNA sequencing, and into M13mp18 (New England Biolabs) for DNA sequencing. Sequencing in M13mp18 was carried out by the dideoxy chain-termination procedure (Sanger, et al., *Proc. Natl. Acad. Sci. USA*, *74*, pages 5463-5467 (1977)) using either modified T7 polymerase

(Sequenase from United States Biochemicals) or Klenow fragment of DNA polymerase (Bio-Rad Laboratories). Priming was performed with either the M13 universal primer or with 17-mer oligonucleotides designed from cDNA sequence. Double-stranded plasmid DNA, isolated by a standard alkaline-lysis mini-prep method (Maniatis, et al., 1982), was sequenced using the method described by Kraft, et al., *Biotechniques*, *6*, pages 544-547 (1988). The plasmid templates were primed with either universal primer, reverse primer (International Biotechnologies, Inc.), or 17-mer oligonucleotides.

Isolation and Characterization of cDNA Clones.

Three clones, positive after tertiary screening, were plaque purified, and phage DNA was prepared. The inserts from the clones, designated lambdaGMPE1, lambdaGMPE2, and lambdaGMPE3, were subcloned into plasmids and sequenced. The DNA sequences of all three clones contained long open reading frames which overlapped. The translated sequence of the longest clone, lambdaGMPE1, contained an amino acid sequence which matched the N-terminal amino acid sequence of intact platelet P-selectin but which lacked an in-frame ATG, which encodes a methionine to initiate translation, 5′ to this sequence.

To identify full-length cDNAs, 1.4 million recombinant phage of the now amplified endothelial-cell cDNA library were rescreened with a 1 kb *Sma*I fragment from the 5′ end of lambda GMPE1. Of the 55 positive clones identified, five were purified. DNA sequence from the 5′ end of one of these new clones, designated lambdaGMPE4, matched the 5′ end of lambdaGMPE1, except that the first 88 bp were not found within the first 140 bp of lambdaGMPE1. Translation of the sequence of lambdaGMPE4 showed

that there was an in-frame ATG sequence beginning at nucleotide 39. The sequence of the 5′ end of lambdaGMPE4 was also found in the other four clones obtained in the second screening of the cDNA library, suggesting that it was the correct sequence and that the first 140 bp of lambdaGMPE1 was a cloning artifact. As shown in Figure 1, sequence ID No.1 is a composite nucleotide sequence of the four clones, with 91% of the sequence derived from lambdaGMPE1. Sequence ID No. 2 is the predicted amino acid sequence.

The composite sequence predicts a 5′ untranslated region of 38 bp, followed by an open reading frame of 2490 bp coding for a protein of 830 amino acids, then a 3′ untranslated sequence of 614 bp including two potential polyadenylation signals of AATAAA and AATTAAA. The latter signal precedes a 12 bp sequence, then a poly(A)$^+$ tail of 75 bp. The nucleotide sequence GxxATGG, surrounding the first in-frame ATG beginning at base 39, agrees with the consensus sequence for initiation of protein translation (Kozak, *Nucl. Acids Res.*, *12*, pages 857-872 (1984)). Therefore the first amino acid was assigned to this codon.

When compared to the sequence of the other clones, there was a 186 bp segment deleted from lambda GMPE1, corresponding to nucleotides 1744 to 1929 (Sequence ID No. 1). A 120 bp deletion was also found in lambda GMPE2 and lambda GMPE3 (nucleotides 2326 to 2445). Eight single-base substitutions (confirmed by sequencing both strands of the cDNAs) were found in the first three clones. Three were silent substitutions, changing the third degenerate base of a codon. The other five produced conservative amino-acid changes.

Northern Blot Analysis.

Total RNA was prepared from the human megakaryocyte-like leukemia cell lines CHRF-288 (Witte, et al., *Cancer*, *58*, pages 238-244 (1986)) and HEL (Papayannopoulou, et al., *J. Clin. Invest.*, *79*, pages 859-866) (1982)), the myeloid cell line K562 (Lozzio and Lozzio, *Blood*, *45*, pages 321-334 (1975)), human umbilical vein endothelial cells, the EA.hy 926 hybrid endothelial cell line (Edgell, et al., *Proc. Natl. Acad. Sci. USA*, *80*, pages 3734-3737 (1983), human platelets, and the Daudi B-cell line (Klein, et al., *Cancer Res.*, *283*, pages 1300-1310 (1969)) by an acid-guanidinium-phenol-chloroform procedure (Chomczynski and Sacchi, *Anal. Biochem.*, *162*:156-159 (1987)). Both HEL cells and K562 cells were treated with Phorbol myristate acetate (PMA) for 48 hr. to induce differentiation before RNA was prepared. Poly(A)$^+$ RNA was isolated from total RNA by oligo-dT cellulose chromatography (Davis, et al., Basic Methods in Molecular Biology (Elsevier, New York 1986). Total or poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel containing formaldehyde, then transferred to a Hybond-N nylon membrane by standard procedures (Maniatis, et al., Molecular Cloning, A Laboratory Manual (Cold Spring Harbor, NY, 1982). The membrane was prehybridized in 5x SSC, 5x Denhardt's solution, 0.2% SDS, and 200 µg/ml herring sperm DNA for oligonucleotide probes, or in 5x Denhardt's solution, 50% formamide, 10% dextran sulfate, and 200 µg/ml herring sperm DNA for cDNA probes. Oligonucleotide probes were labeled by the procedure described above, and cDNA probes were random-labeled with a α-^{32}P dCTP using the Klenow fragment of DNA polymerase in a commercial kit (Boehringer-Mannheim Biochemicals). Probes were hybridized overnight at 42°C at a specific activity of at least 1 x 10^6

cpm/ml. The filters to which the oligonucleotides were hybridized were washed with 2x SSC, 0.5% SDS for 20-min periods at increasing temperatures up to 52°C, whereas those used with the cDNA probes were washed with 0.2x SSC, 0.1% SDS at temperatures up to 60°C and were exposed to film (X-OMAT AR, Kodak) at -80°C. Molecular weight markers of lambda DNA (Boehringer-Mannheim Biochemicals), electrophoresed in parallel with the RNA samples, were visualized by including labeled lambda/*Hin*dIII DNA fragments (Bethesda Research Laboratories) [5 x 10^5 cpm/ml] in the hybridization solution.

Southern Blot Analysis.

Human placental genomic DNA (intact or digested with *Eco*RI, *Bam*HI, and *Bam*HI/*Hin*dIII) were obtained from Oncor. Intact DNA was also digested with *Pst*I, *Taq*I, and *Xba*I. Ten μg of each digested DNA was electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane using a modification of the procedure of Reed and Mann, *Nucl.Acids Res.*, *13*, pages 7207-7221 (1985). Briefly, the gel was soaked in 0.2 M HCl for 10 minutes, rinsed four times in water, and transferred to a nylon membrane in 0.4 M NaOH buffer for 1 hr, then in 20x SSC overnight. The DNA was fixed to the membrane by exposure to UV light and hybridized with cDNA as described for the Northern blot. Lambda/*Hin*dIII fragments and ΦX174/*Hae*III fragments (Bethesda Research Laboratories) were used as standards and were visualized by including randomly labeled DNA fragments of lambda and ΦX174 (5 x 10^5 cpm/ml) in the hybridization solution.

Computer Analysis.

DNA and protein sequence were analyzed using the Genetics Computer Group software package of the University of Wisconsin (Devereux, et al.,

Nucl.Acids Res., *12*, pages 387-395 (1984)). The amino acid sequence of P-selectin was compared with the published sequences of other proteins contained in the National Biomedical Research Foundation (NBRF) database (Release 16.0; 3/88).

The 5′ Flanking Region of the Human P-selectin Gene

The following methods were used for the isolation and characterization of the 5′ flanking region of the P-selectin gene.

Cells.

Human HL-60 promyelocytic cells (ATCC No. CCL240), K562 erythroid cells (ATCC No. CCL 243), HEL erythroleukemia cells (ATCC No. TIB180), and Jurkat T-lymphocytes were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Human HeLa epithelioid cells (ATCC No. CCL 2), 293 human embryonal kidney cells (ATCC No. CRL 1573), and COS-7 SV40-transformed African green monkey kidney cells (ATCC No. CRL 1651) were maintained in Dulbecco's Minimal Essential Medium (DMEM, high glucose) supplemented with 10% FCS. CHRF-288 human megakaryocytic cells (Witte et al., *Cancer*, *58*, pages 251-256 (1986)) were maintained in Fisher's medium supplemented with 20% horse serum. Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were cultured as previously described (Moore et al., *J. Clin. Invest.*, *79*, pages 124-130 (1987)).

Genomic cloning and Southern blot analysis.

A human genomic clone designated EMBL3-1 was obtained by screening a human genomic DNA library with a ^{32}P-labeled P-selectin cDNA as described above and by Johnston et al., *J. Biol. Chem.*, *265*, pages 21381-21385 (1990). DNA restriction fragments derived from this clone were subcloned into the plasmid pIBI20 (IBI) for restriction enzyme mapping and DNA sequencing. All sequencing

was carried out by the chain-termination procedure (Sanger et al., *Proc. Natl. Acad. Sci. USA, 74,* pages 5463-5467 (1977)) using Sequenase (United States Biochemicals). Southern blot analysis of human placenta genomic DNA was done as previously described (Johnston et al., *Cell, 56,* pages 1033-1044 (1989)) using a random-labeled probe corresponding to the 5′ flanking region of the P-selectin gene from nucleotides (nt) 4405 to 4842 of SEQ ID NO. 5.

Determination of transcriptional start sites.

Poly(A)$^+$ RNAs were prepared from cultured HUVEC, CHRF-288, HEL, and HL-60 cells by using the Fasttrack kit (Invitrogen). The poly(A)$^+$ RNAs were used for primer extension studies, RNase protection experiments, and anchored polymerase chain reaction (PCR) cloning. For primer extension analysis, a 27-mer primer was prepared with the sequence, 5′-TTCTGGTTTGTTAGTTCAGAGATCAGG-3′(SEQ ID NO. 6). The primer was 5′ end-labeled with [γ-^{32}P]ATP (New England Nuclear, Inc) using T4 polynucleotide kinase (Pharmacia) and purified twice through a RNase free G-25 spin column (5′-3′ Inc.). The specific activity of the labeled primer was 4 x 10^6 cpm/pmol. The annealing of the primer and the extension reaction were performed as described by Mackman et al., *Proc. Natl. Acad. Sci. USA, 87,* pages 2254-2258 (1990) except that actinomycin D was not added. The reaction products were then extracted with phenol/chloroform, precipitated by ethanol, and separated on a 6% sequencing gel along with a known DNA sequencing ladder.

To confirm the results obtained by primer extension analysis, RNase protection studies were carried out with two different cRNA probes by the method of Cox et al., *Blood,* 77, pages 286-293 (1991). Briefly, two genomic DNA fragments

spanning the region from nt 4555 to 4842 and nt 4405 to 4842, respectively, of SEQ ID NO. 5 w re amplified by PCR and subcloned into plasmid pIBI20 which contains a T7 promoter. The insert nucleotide sequences were confirmed. The recombinant plasmids were linearized with an appropriate restriction enzyme and the cRNA probes were generated with T7 RNA polymerase (Promega). The cRNA probes were then purified on a 6% sequencing gel. The linearized plasmids and cRNA probes were hybridized in 4 M guanidine thiocyanate and 25 mM EDTA (pH 6.0) at 42°C overnight, followed by treatment with RNase A and RNase T1 (Promega). Analysis of the protected products was performed as described for the primer extension studies.

As a definitive approach to determine the transcriptional start sites, anchored PCR (Frohman et al., *Proc. Natl. Acad. Sci. USA, 85,* pages 8998-9002 (1988) was used to clone the 5′ ends of a number of P-selectin cDNAs. First-strand cDNA was synthesized from 2 μg of HEL cell mRNA with the cDNA Cyclekit (Invitrogen), containing the strong RNA denaturant methylmercuric hydroxide (MeHgOH), with a specific primer, 5′-GATGTATATCTCCACGCAGTCCTCG-3′ (SEQ ID NO. 7), which is complementary to nt 446-422 of SEQ ID NO. 3. After removing the excess primer with a Centricon 100 spin filter (Amicon), the 3′ end of the first strand cDNA was tailed in a 30-μl volume containing tailing buffer (Bethesda Research Laboratories), 1 mM dATP, and 15 units of terminal deoxynucleotidyl-transferase (Bethesda Research laboratories) for 10 min at 37°C, and then heated for 15 min at 65°C. The reaction mixture was diluted to 200 μl, and 10-μl aliquots were used to synthesize the second strand cDNA with 10 pmol of the anchored-primer, 5′-GAATTCGAGCTCGGTACC

TTTTTTTTTTTTTTTT-3′(SEQ ID NO. 8), using 2.5 units of Taq DNA polymerase (Cetus) at 72°C for 7 min. The mixture was then subjected to PCR with two additional primers to improve specific amplification and facilitate subcloning: an adaptor primer, 5′-GAATTCG AGCTCGGTACC-3′(SEQ ID NO. 9), which corresponded to the 5′ end of the anchored primer and included restriction sites for EcoRI, SacI and KpnI, and a nested primer 5′-GTCGACTCTAGAATCAGCCCAGTTCTCAGC-3′ (SEQ ID NO. 10), which was complementary to nucleotides 378-395 of the cDNA sequence and included *XbaI* and *SalI* sites. PCR was performed in a Perkin-Elmer/Cetus thermal cycler; the amplification profile involved denaturation at 94°C for 1.5 min, primer annealing at 55°C for 2.5 min, and extension at 72°C for 1 min. The anchored PCR products were detected by Southern blot analysis with two ^{32}P-labeled internal oligonucleotides to verify the specificity of the reaction. The largest products were then subcloned into pIBI20, and plasmid inserts from 21 individual colonies were sequenced.

Construction of chimeric luciferase expression vectors.

Plasmid p0LUC (originally designated p19LUC) and pRSVLUC (DeWet et al., *Mol. Cell. Biol.*, *7*, pages 725-737 (1987)) were gifts from Dr. Donald Helinski (University of California, San Diego). The parental DNA for the creation of deletion mutants was a 5-kb *Hind*III fragment inserted into pIBI20 (pGHindIII, Figure 2) that contained the 5′ flanking region, first exon, and part of the first intron of the P-selectin gene. Plasmid p1339LUC in the correct orientation and p1339RLUC in reverse orientation were constructed by inserting the 1.3 kb P-selectin 5′ flanking region excised by *HinfI* from pGHindIII into the *SmaI* site of p0LUC. Plasmids p701LUC, p701RLUC, p459LUC, p309LUC,

p249LUC, p197LUC, p147LUC, p128LUC, p100LUC, and p80LUC were constructed by ligation of the respective DNA segments generated by PCR from pGHindIII into the *SmaI* site of p0LUC. Plasmid p4863LUC was constructed in three steps: 1) ligation into the *EcoRV* and *EcoRI* sites of pBluescript II KS (Stratagene, La Jolla, CA) of the fragment from nt 4596 to 4851 of SEQ ID NO. 5 released from p309LUC with *EcoRV* and *EcoRI*; 2) insertion between the *HindIII* and *EcoRV* sites of the above construct of a fragment from nt 1 to 4596 of SEQ ID NO. 5 released from pGHindIII with *HindIII* and *EcoRV*; and 3) removal by *HindIII* and *SmaI* of the sequence from nt 1 to 4851 of SEQ ID NO. 5 from the above construct followed by ligation into the *HindIII* and *SmaI* sites of p0LUC. Plasmid pm309LUC, pm249LUC, and pm197LUC, each of which contained three identical mutations in the TTATCA element, were constructed by an overlap extension PCR protocol (Disdier et al., *Mol. Biol. Cell*, *3*, pages 309-321 (1992)). Briefly, two separate PCR products, one for each half of the hybrid product, were generated with either an antisense or sense mutated GATA oligonucleotide (described below for the gel shift assay) and one outside primer. The two products were gel purified and mixed. A second PCR was then performed using the two outside primers. The PCR product was blunt-ligated into the *SmaI* site of p0LUC. All the constructs were verified by sequencing the inserts and flanking sites in the plasmid.

Molecular cloning of the 5′ flanking region of the P-selectin gene.

The genomic clone EMBL3-1 encoding the 5′ untranslated region of P-selectin cDNA was obtained, as described above, by screening a genomic DNA library with a ^{32}P-labeled P-selectin cDNA (Johnston et al., *J. Biol. Chem.*, *265*, pages

21381-21385 (1990)). A 5 kb HindIII fragment encoding the 5-untranslated region of the P-selectin gene, derived from genomic clone EMBL3-1, was subcloned into pIBI20, analyzed by restriction mapping (Figure 2), and sequenced on both strands. Southern blot analysis of human genomic DNA with a labeled DNA fragment derived from this clone revealed that restriction fragments were identical in size to those in the clone, suggesting that there was no DNA rearrangement during cloning.

Transcriptional start sites in the 5′ flanking region.

To define the transcriptional start sites for the P-selectin gene, three assays were used, as shown in Figure 3.

To map transcriptional start sites by primer extension, 10 μg of poly (A^+) RNA from HEL cells, CHRF-288 cells, or HUVEC, or 10 μg of yeast tRNA was hybridized with a 5′-end-labeled oligonucleotide primer. After incubation with reverse transcriptase, primer-extended products were analyzed on a 6% sequencing gel. A sequence ladder of the plasmid pIBI20 was used as size markers (lanes G, A, T, and C). At least 12 extension products were seen from nt 4769 to 4839 of SEQ ID NO. 5 relative to the ATG codon initiating translation of mRNA. Extension products of the same size were produced by mRNA from HUVEC and from the megakaryocytic cell lines CHRF-288 and HEL, but not by control tRNA.

Similar results were obtained by RNase protection assay using two independent cRNA probes surrounding the first exon. The two cRNA probes produced the same protected band patterns, most of which corresponded in size to the primer extension products. Ten micrograms of poly (A^+) RNA from HEL cells, HL-60 cells, or yeast tRNA were hybridized with each of the two different cRNA probes. After

treatment with RNase, the protected products were run on a 6% sequencing gel. RNase-protected bands corresponding to the smallest primer extension products were not observed due to the short cRNA probes used. However, these short primer extension products were consistently observed, indicating that they reflected alternative transcriptional start sites rather than premature stops during reverse transcription.

To rule out generation of multiple bands through alternative splicing of pre-mRNAs, anchored PCR was performed to clone the 5′ ends of cDNAs from HEL cell mRNA. Twenty-one clones were sequenced. The sequences of the 5′ ends of these cDNA clones matched the genomic sequence and corresponded in length to those predicted from the primer extension and RNase protection products.

The most abundant clones began at 4786, 4814, and 4832 (four clones each) of SEQ ID NO. 5, which matched the sizes of the most prominent products determined by RNase protection and/or primer extension. These data, summarized in Figure 4, indicate that transcription of the P-selectin gene is initiated at multiple identical start sites in both endothelial and megakaryocytic cells.

Structural features of the 5′ flanking region.

The sequence of 4866 bp of 5′ flanking region concluding with the ATG at the end of the exon 1 is shown in SEQ. ID NO:3. No canonical TATA and CCAAT boxes were found in the P-selectin flanking sequence. The nucleotide sequence was not GC rich and lacked a GC box (the binding site for transcription factor Sp1) that is found in many "housekeeping" genes without TATA boxes (Reynolds et al., *Cell*, *38*, pages 275-285 (1984)). It also lacked an "initiator" sequence which is required for accurate initiation of transcription in some

TATA-less genes (Smale et al., *Cell*, *57*, pages 103-113 (1989)). A number of potential regulatory elements were present, including two sites at 4707-4711 and 4105-4110 recognized by the GATA family of zinc finger transcription factors (Yamamoto et al., *Genes & Dev.*, *4*, pages 1650-1662 (1990); Orkin, *Cell*, *63*, pages 665-672 (1990), a CACCC (GGGTG) element at 4648-4652 frequently seen in the promoters of erythroid-expressed genes (Frampton et al., *Mol. Cell. Biol.*, *10*, pages 3838-3842 (1990); Schule et al., *Nature*, *332*, pages 87-90 (1988), a GGGGGTGACCCC (4646-4657 of SEQ ID No. 5) overlapping with the CACCC element that is similar to the binding sites recognized by the subunit NFKB1 (p50) of the NF-κB/rel family (Blank et al., *Trends Biochem Sci.*, *17*, pages 135-140 (1992)) and by a zinc finger nuclear protein family that includes MBP-1 and MBP-2, 10 elements beginning at 4761, 4727, 4641, 4645, 4505, 4471, 4457, 4212, 4206, and 3732 of SEQ ID NO. 5, which contain a GGAAG/A SEQ core sequence that is similar to the binding site for the ETS class of oncoproteins (Karim et al., *Genes & Dev.*, *4*, pages 1451-1453 (1990)), and a TCTGGAATGTG (4747-4757 of SEQ ID NO. 5) that is related to the GT-IIC element of the SV40 enhancer (Burglin, *Cell*, *66*, pages 11-12 (1991)).

II. <u>Cell Specific Expression under the control of the 5′ P Selectin Regulatory Sequence.</u>

<u>Transfection and Luciferase assay</u>.

Plasmids used for transfections were purified by cesium chloride banding. At least two different batches of plasmids for each construct were tested for the transfections. Cells were plated on a 100 mm petri dish at a density adjusted so that they reached 70-80% confluence prior to transfection. Equal volumes of 60 μg of test plasmid and 50 μg of lipofectin reagent (BRL), each diluted in 2.5 ml of

OptiMEM medium (BRL), were incubated for 20 min and the resulting transfection mixture was then added to the cells. After an 8 to 12 h incubation at 37°C, the transfection medium was replaced by complete medium for an additional 36 h and the cells were then harvested for luciferase assays in a total volume of 120 μl (DeWet et al., *Mol. Cell. Biol.*, *7*, pages 725-727 (1987)). Briefly, following lysis and removal of the cell debris by centrifugation, 20 μl of total cellular extracts were used for each measurement for luciferase activity. The luciferase activity for each transfection was measured three times with a Monolight 2001 luminometer. The luciferase activities were normalized to the amount of protein in cellular extracts as measured by the Bradford reagent (Bio-Rad).

Preparation of cell extracts and gel shift assay.

HEL, CHRF-288, K562, Jurkat, Hela, and BAEC cell nuclear extracts were prepared as described by Dignam et al., *Nucl. Acids Res.*, *11*, pages 1475-1489 (1983). Extracts from HUVEC were prepared at miniscale as described by Schreiber et al., *Nucl. Acids Res.*, *17*, pages 6419 (1989). COS-7 cells were transfected with a plasmid encoding human GATA-2 (Dorfman et al., *J. Biol. Chem.*, 267, pages 1279-1285 (1992)), a gift from Dr. Stuart Orkin (Harvard Medical School, Cambridge, MA), or mock-transfected with the plasmid pIBI20. Extracts from GATA-2-transfected or mock-transfected COS-7 cells were prepared as described in Tsai et al., *Nature*, *339*, pages 446-451 (1989). A standard gel shift assay (20 μl) contained 5,000-10,000 cpm of labeled oligonucleotide, 2.5 μg of poly(dI·dC), 60 mM KCl, 4 mM Tris (pH 7.5), 12 mM Hepes, 1 mM dithiothreitol, 1 mM EDTA, 10 μg bovine serum

albumin, and 3 μl (6 μg) of cell extracts. Gels of 4-6% acrylamide (19:1 acrylamide/N,N'-methylenebis- acrylamide w:w) were run in 0.25 x TBE buffer (1x TBE = 0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA) at 150 V and then dried prior to autoradiography. For competition experiments, unlabeled competitor duplex DNA was added to the reaction mixture before the addition of labeled probe.

Transgenic Mice.

The *lacZ* gene was excised from pCH110 (Pharmacia-LKB) by digestion with *Hin*dIII and *Apa*I and ligated into the phagemid, pBluescript SK+ (Stratagene), pre-digested with the same enzymes. Constructs 1-3 were prepared by removing the 5' flanking sequences of the P-selectin gene from, respectively, p1339LUC, p1339RLUC, and p701LUC (see above), by digestion with BamHI and XbaI and inserting the sequences into pBluescript at the corresponding sites such that the 5' flanking sequence was separated from *lacZ* by only a few base pairs of sequence in the polylinker region of the plasmid. Constructs 4 and 5 were prepared by excising the 5' flanking sequences from p459LUC and p309LUC with HindIII and EcoRI and inserting the sequences into the same *lacZ*-Bluescript vector.

Each of the five plasmids was purified on cesium chloride gradients. The insert containing the P-selectin 5' flanking region fused to *lacZ* was released from the plasmid by digestion with BamHI and XbaI. After purification from agarose gels, the DNA insert was microinjected into the pronuclei of oocytes obtained from mating mice. The microinjected oocytes were then implanted into the infundibulum of the Fallopian tubes of pseudopregnant mice using standard techniques (Hogan et al. in Manipulating the Mouse Embryo. A

Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986) (incorporated herein by reference). Incorporation of the transgene into offspring was determined by PCR analysis of tail-vein DNA, using primers specific for the 5′ flanking sequence and the *lacZ* sequence (Chen and Evans, *Biotechniques*, *8*, 32-33 (1990)). Expression of the transgene in tissues of founder mice or their offspring was determined by a cytochemical staining procedure for β-galactosidase (Sanes et al., *EMBO J.*, *5*, pages 3133-3142 (1986)).

Transient expression analysis of the 5′ flanking region.

To test whether the 5′ flanking region of the P-selectin gene had promoter activity, chimeric constructs were prepared in which serially deleted fragments of 5′ flanking sequence were inserted before a promoterless luciferase gene in the plasmid p0LUC (Figure 5). Luciferase expression was measured following transfection of the constructs into BAEC. Constructs p4863LUC, p1339LUC, p701LUC, p459LUC, p309LUC, and p249LUC promoted similar levels of luciferase activity that were significantly higher than the background amounts in p0LUC-transfected cells. Serial decreases in expression were observed following transfection with p197LUC and with p147LUC and p128LUC. Only background expression was observed following transfection with p100LUC and p80LUC. These data indicate that the sequences responsible for most of the promoter activity are located between 4615 and 4861 of SEQ ID NO. 5. These data also indicate that there are at least three positive regulatory domains between 4615 and 4764 of SEQ ID NO. 5 (for example, compare p249LUC, p197LUC, p128LUC, and p100LUC). The results also show that some of the positive elements in the longer constructs were position dependent, as

p1339RLUC and p701RLUC, which contained flanking sequence in reverse orientation, expressed no more luciferase in BAEC than p0LUC.

To determine whether the constructs mediated tissue-specific expression, they were transfected into COS-7, 293, and Hela cells, which do not normally synthesize P-selectin, as shown by Figure 6. In contrast to their effects in BAEC, constructs p1339LUC, p701LUC, p459LUC, and p309LUC had only basal promoter activity in these cells, although the basal levels did exceed the background activity of mock-transfected cells or cells transfected with p0LUC. Parallel transfections with RSVLUC, a plasmid containing luciferase driven by the Rous sarcoma virus promoter, resulted in high levels of expression, indicating that the cells could be transfected. These data indicate that the flanking sequence from nt 4555 to 4851 of SEQ ID NO. 5 includes elements that can direct the regulated expression of a gene in endothelial cells.

The GATA element at 4706 of SEQ ID NO. 5 is required for optimal function of the P-selectin promoter.

Deletion of the sequence between nt 4667 and 4717 of SEQ ID NO. 5 significantly reduced luciferase expression in BAEC, as shown by Figure 5, indicating the presence of a positive regulatory element(s) in this region. To determine whether the GATA (TTATCA) element at 4706 of SEQ ID NO. 5 functioned as such a positive element, the wild-type sequence TTATCA was mutated to TTTAGA in the three constructs p309LUC, p249LUC, and p197LUC, as shown by Figure 6. When the mutant constructs were transfected into BAEC, luciferase expression in each case was reduced to the level of p147LUC, which was only 20% of that produced by p309LUC. These results indicate that the GATA element is

essential for optimal transcription of the P-selectin gene, perhaps through interactions with other regulatory sequences located between nt 4555 to 4666 of SEQ ID NO. 5.

Gene Expression in Megakaryocytes of Transgenic Mice Containing the P-selectin Promoter.

The use of the 5′ flanking region of the P-selectin gene to direct the expression of a heterologous gene in megakaryocytes was demonstrated by analysis of transgenic mice carrying portions of the 5′ flanking region of the P-selectin gene fused to the bacterial *lacZ* gene.

Five constructs were made in which DNA, having the sequence of nt 3525 to 4851 (or the same sequence inserted in the opposite orientation), 4163 to 4851, 4405 to 4851, or 4555 to 4851 of SEQ ID NO. 5, was fused to the 5′ coding region of the *lacZ* gene, as described in Figure 9. Each construct was then cloned and used to prepare transgenic mice as described above. Offspring carrying the transgenes for each of the five constructs were produced from founders. An analysis of bone marrow cells from mice carrying the construct having the sequence of nt 4163 to 4851 of SEQ ID NO. 5 fused to *lacZ* indicated that the transgene was expressed by megakaryocytes but not by other bone marrow cells.

The above results clearly indicate that the sequences of the 5′ flanking region of the P-selectin gene can specifically direct the regulated expression of a gene in endothelial and megakaryocytic cells. Accordingly, a gene can be specifically expressed in endothelial cells or megakaryocytes by ligating, or otherwise fusing (for example, by PCR), the 5′ end of the coding sequence of the gene to the 3′ end of a DNA sequence consisting essentially of nucleotides 1 to

4863 of SEQ ID NO. 5, or one or more of the transcriptional regulatory sequences of SEQ ID NO. 5, to yield a recombinant gene construct, and transfecting, or otherwise inserting using methods known to those skilled in the art (for example, by transgenic methods, microinjection, liposome fusion) the recombinant gene construct into endothelial cells or megakaryocytes.

It is likely that some modifications in the sequence of the 5′ flanking region can be introduced into the cells without alteration in function. For example, the 5′ flanking sequences of a given gene in different species may be slightly different; the differences are usually in regions not critical for function and therefore not conserved among species. It is believed that the 5′ flanking sequences of the P-selectin gene in other species will also be functional in humans; this prediction is supported by the observed function of the human P-selectin flanking sequence in transgenic mice (see above).

III. **The 5′-P Selectin Regulatory Sequence can be used to isolate Novel Proteins.**

The function of the GATA element correlates with its ability to bind nuclear proteins.

To determine whether the GATA element bound nuclear proteins, a 40-bp double-stranded oligonucleotide probe encompassing this sequence was synthesized, as shown in Figure 8. The ^{32}P-labeled probe was incubated with nuclear extracts from various cell lines in the presence of poly (dI•dC) as a competitor for nonspecific DNA-protein interactions. The resultant complexes were separated by electrophoresis on a nondenaturing polyacrylamide gel. For example, two distinct complexes (labeled A and B, where A has the slower mobility in the gel) were observed on gels when K562 and HEL cell nuclear extracts were incubated

with end-labeled P-selectin GATA oligonucleotide. Complex formation was sequence specific, as it was prevented by addition of a 100-fold molar excess of the unlabeled GATA oligonucleotide, but not by a 100-fold excess of an oligonucleotide encoding a nonconsensus GATA sequence located further upstream in the P-selectin 5′ flanking region. Similar complexes were formed using nuclear extracts from CHRF-288, BAEC, HUVEC, and Jurkat cells, although sometimes one of the complexes was only present in relatively small amounts. Two minor complexes were also observed in the gels, but these appeared to represent proteolytic degradation products as they were noted only in nuclear extracts stored for prolonged intervals.

To determine whether the GATA element was required to form the observed complexes in the gel shift assays, competition gel shift assays were carried out on HEL cell nuclear extracts with an oligonucleotide encoding the GATA motif from the human endothelin-1 gene promoter and the results compared to a competition gel shift assay carried out with the oligonucleotide encoding the P-selectin GATA motif, as shown by Figure 8. The results indicated that the unlabeled human endothelin-1 GATA oligonucleotide probe prevented formation of complex B, but not complex A, by the ^{32}P-labeled P-selectin probe containing the GATA consensus sequence. The labeled endothelin-1 probe formed a complex with a mobility similar, although not identical, to complex B. Formation of this complex was prevented by addition of either the unlabeled endothelin-1 probe or the P-selectin probe.

These data indicate that complex B represents the interaction of a member of the GATA protein

family with the GATA consensus sequence in the P-selectin promoter.

To test whether the core GATA sequence was required for binding of nuclear proteins, a mutant oligonucleotide was synthesized (Figure 8) in which the core sequence TTATCA was converted to TTTAGA, the same changes made in the mutant constructs shown in Figure 7. HEL cell and BAEC nuclear extracts were incubated with end-labeled wild-type or mutant P-selectin GATA probe in the absence or the presence of the indicated unlabeled competitor. The results showed that a 100-fold molar excess of the unlabeled mutant probe prevented formation of complex A, but not complex B, when the labeled P-selectin GATA oligonucleotide was incubated with nuclear extracts from HEL and BAEC. Furthermore, the labeled mutant probe formed complex A, but not complex B. These data indicate that the core GATA sequence is required for formation of complex B, but not complex A.

To confirm that the consensus GATA element in the P-selectin promoter bound to a member of the GATA protein family, gel shift assays were performed with extracts from COS-7 cells transfected with an expression plasmid encoding human GATA-2. Labeled P-selectin probe was incubated with extracts from mock-transfected COS-7 cells (mock) or with COS-7 cells transfected with an expression plasmid encoding human GATA-2 (hGATA-2) in the present or absence of unlabeled P-selectin GATA, unlabeled human endothelin-1 GATA, or nonconsensus GATA oligonucleotides as competitors.

The hGATA-2-transfected COS-7 extracts, but not those from mock-transfected cells, formed a DNA-protein complex with the ^{32}P-labeled P-selectin GATA probe. Formation of this complex was

inhibited by addition of the unlabeled endothelin-1 GATA oligonucleotide as well as by the unlabeled P-selectin probe. These results indicate that the P-selectin GATA probe binds a member(s) of the GATA protein family.

Other nuclear protein binding sites.

The luciferase expression studies described above indicated that the sequences responsible for most of the P-selectin promoter activity in endothelial cells are located in the sequence between nt 4615 and 4851 of SEQ ID NO. 5 and that at least three positive regulatory sequences are located between nt 4615 and 4764 of SEQ ID NO. 5. In addition to these sites, gel shift studies also indicated the existence of at least three regulatory sites in a 52 base pair portion of the 5′ flanking region of the P-selectin gene having the sequence from nt 4615 to 4666 of SEQ ID NO. 5. This conclusion was obtained by gel shift studies of four complexes that were first observed to form between nuclear protein extracts from several cell types and a double-stranded ^{32}P-labeled oligonucleotide corresponding to the sequence from nt 4632 to 4672 of SEQ ID NO. 5. Starting with the complex with slowest mobility in the gels, these complexes were designated as I, then a closely-spaced doublet IIa and IIb, then III. Extracts from some cell types formed some, but not all, of the complexes. For example, complex I was not formed by extracts of HUVECs.

Complex I was also formed by mixing nuclear extracts with an oligonucleotide having the sequence of nt 4650 to 4669 of SEQ ID NO. 5. This sequence is not related to those of known DNA regulatory elements. Complex I was formed with nuclear extracts from BAECs, but complex formation was prevented when extracts were prepared from the

same cells following stimulation with phorbol myristate acetate. These results indicate that the sequence of nt 4650 to 4669 of SEQ ID NO. 5 is bound by previously undescribed regulatory protein(s).

The complexes corresponding to the IIa and IIb doublet (referred to collectively as complex II for simplicity) represent interactions of the DNA with members of the NFκB family, a group of homodimeric or heterodimeric DNA-binding proteins that help regulate expression of many genes (Blank et al., *Trends Biochem. Sci.*, *17*, pages 135-140 (1992). Complex II was formed not only when nuclear extracts were mixed with the oligonucleotide having the sequence of nt 4632 to 4672 of SEQ ID NO. 5, but also when mixed with a oligonucleotide having the shorter sequence of nt 4642 to 4664 of SEQ ID NO. 5. Formation of complex II was inhibited by an unlabeled oligonucleotide having the sequence CGGCTGGGGATTCCCCATCT (SEQ ID NO. 11), which contains the NFκB element of the mouse *H-2K*^{b} class I major histocompatibility (MHC) promoter. Complex formation was also inhibited by a combination of antisera to NFKB1 and NFKB2 subunits, two of the subunits found in certain dimeric NF-KB proteins, but not by preimmune sera. Furthermore, the labeled oligonucleotides bound to purified recombinant homodimers of NFKB1 and NFKB2, but not to homodimers of recombinant Rel A, another subunit found in some dimeric NF-KB proteins, or to heterodimers that contain the Rel A subunit in cell extracts. Methylation interference studies indicated that the GGGG sequence in the sense strand (nt 4646 to 4650 of SEQ ID NO. 5) and the GGGG in the antisense strand (nt 4654 to 4657 of SEQ ID No. 5) participated in direct contacts with NFKB1 and NFKB2 homodimers. Furthermore, a mutant

oligonucleotide spanning the 4642 to 4664 sequence, in which the CCCC sequence at 4654 to 4657 was altered to ATAG, failed to interact with NFKB1 or NFKB2 in gel shift assays. These results indicate that the sequences recognized by NKFB1 and NFKB2 in the 4642 to 4664 region are consistent with known recognition features for members of the NF-κB family.

Formation of complex III required the inverted repeat sequence of nt 4635 to 4646 of SEQ ID NO. 5. Simultaneous mutations in both halves of the inverted repeat changing the sequence from CTTCCATGGAAG (nt 4635 to 4646 of SEQ ID NO. 5) to GTTGGTTCCAAG (SEQ ID No. 12), GTTGGTTTTAAG (SEQ ID No. 13), or CTTCCATCCAAG (SEQ ID No. 14), abolished complex formation. An oligonucleotide having the sequence from nt 4626 to 4650 of SEQ ID NO. 5 also formed complex III. The sequence requirements for the formation of complex III indicate that one or more members of the ETS class of transcription factors may bind to this sequence of the 5' flanking region of the P-selectin gene. Complex III was formed by extracts from all cells tested (HEL, CHRF-288, K562, Jurkat, HL-60, HUVEC, BAEC, EAhy.926, and Hela), indicating that the transcription factor(s) interacting with sequence of 4626 to 4650 of SEQ ID No. 5 is widely expressed. Of the known ETS proteins, ets-2 and GABP are the most widely distributed (Macleod et al., *Trends Biochem. Sci.*, 17, pages 251-256 (1992)). However, oligonucleotides encoding known recognition sites for these proteins, that is, TCGAGCAGGAAGTGACGTCGA (SEQ ID NO. 15) or GGCCAAACCGGAAGCATGTG (SEQ ID NO. 16) for ets-2 and AGCTTGCGGAACGGAAGCGGAAACCGCCGGATCG (SEQ ID NO. 17) for GABP, did not prevent formation of complex III with the labeled -238 to -214 oligonucleotide,

indicating that ets-2 and GABP are not interacting with this region in the 5′ flanking region of the P-selectin gen . These results indicate that the portion of the 5′ flanking region of the P-selectin gene defined by nt 4635 to 4646 of SEQ ID NO. 5 is bound by previously undescribed protein(s). Such proteins may be unidentified members of the ETS family or of other families of transcription factors.

Recently a pathway has been elucidated that connects signaling at the cell surface directly to gene activation (reviewed by Darnell, et al., *Science, 264,* pages 1415-1421 (1994); Ihle, et al., *Trends Biochem. Sci., 19,* pages 222-227 (1994); Sato and Miyajima, *Curr. Opin. Cell Biol., 6,* pages 174-179 (1994)). This pathway is used by the interferons and by many cytokines and growth factors, including IL-3, IL-4, IL-6, oncostatin M, and epidermal growth factor. In general, binding of ligand induces dimerization or oligomerization of its receptor that allows association with JAK kinases, which phosphorylate a tyrosine residue in a group of transcription factors known as the STATs (signal transducers and activators of transcription). The phosphorylated STATs then dimerize and migrate to the nucleus, where they bind to specific DNA elements, alone or in combination with other transcription factors, resulting in transciptional activation. Cytokines IL-3, IL-4, IL-6, and oncostatin M induce increased levels of P-selectin mRNA in cultured human umbilical vein endothelial cells or in bovine aortic endothelial cells, as measured by Northern blotting. Transcript levels begin to increase at 7 hours and are even higher at 24 hours following stimulation with each cytokine. A review of the sequence of the 5′ flanking region of the human P-

sel ctin gene revealed at least four elements that shared sequence similarity to consensus binding sites for members of the STAT protein family: nt 4453 to 4461, nt 4636 to 4645, nt 4722 to 4731, and nt 4745 to 4753 of SEQ ID NO. 5. A double-stranded oligonucleotide encompassing the first putative element, consisting of the sequence from nt 4446 to 4469 of SEQ ID NO. 5, formed inducible complexes with nuclear proteins from several cell lines stimulated for 30 min with interferon-γ or IL-6. Formation of these complexes was inhibited by addition of an unlabeled oligonucleotide containing the hSIE sequence, an element known to bind with high affinities to stat1 homodimers, stat3 homodimers, and stat1-3 heterodimers (Zhong, et al., *Science, 264*, pages 95-98 (1994)). Antibody supershift experiments confirmed that two of the complexes formed by the SEQ 18 oligonucleotide contained stat1, and the mobility of another complex, elicited primarily by IL-6, suggests that it represents stat3 homodimers. The SEQ 18 element formed two additional complexes with nuclear extracts from the human megakaryocytic cell line, CHRF-288; one of these complexes was inducible by treatment with IL-4 for 16 hours. Formation of the latter two complexes, which may represent binding to novel STAT proteins, was not competed with the hSIE oligonucleotide. Collectively, these data indicate that several different cytokines can induce increased steady-state levels of P-selectin transcripts, and that one potential mechanism for these inducible increases may be binding of phosphorylated STAT proteins to elements in the 5′ flanking region of the human P-selectin gene.

The gel-shift assays clearly indicate that sequences defined by nt 4650 to 4669, 4635 to 4646, and 4642 to 4664 of SEQ ID NO. 5, identify distinct

domains within in the 5′ flanking region of the P-selectin gene which play a significant role in the regulation of the expression of the P-selectin gene in endothelial and megakaryocytic cells. The gel-shift assays also indicate the existence of previously unknown regulatory proteins that bind these distinct domains of the 5′ flanking region of the P-selectin gene. In particular, sequences defined by nt 4650 to 4669 and nt 4635 to 4646 of Seq ID No. 5 were sufficient to form two distinct complexes, i.e., complex I and complex III, respectively. Unlike the sequence defined by nt 4642 to 4664 of SEQ ID NO. 5, which has recognition features for proteins of the NFkB regulatory protein family, the sequences required for complex I have no obvious homology to sequences recognized by any known regulatory protein family. The sequences required to form complex III may allow binding of an unknown member of the ETS family or binding of a previously uncharacterized protein in some other class of transcription factors. The nucleotide sequence defined by nt 4446 to 4469 of SEQ ID NO. 5 appears to bind two known STAT family members, stat1 and stat3, and may bind to additional novel members of this family. Elements encompassing nt 4636 to 4645, nt 4722 to 4731, and nt 4745 to 4753 of SEQ ID NO. 5 are also candidate binding sites for known or novel STAT proteins.

Accordingly, the sequences of the 5′ flanking region of the P-selectin gene defined by nt 4650 to 4669 and 4635 to 4646 of SEQ ID NO. 5 are recognized and bound by regulatory proteins with previously undescribed features and are useful for the identification and isolation of such regulatory proteins. Sequences encompassing nt 4446 to 4469, nt 4636 to 4645, nt 4722 to 4731, and nt 4745 to 4753 of SEQ ID NO. 5 may also bind to novel members

of the STAT family of transcription factors and could be used for th identification and isolation of such proteins. For example, such sequences could be attached to resins for use in the isolation of unknown DNA binding proteins by affinity chromatography. As another example, such sequences could be labeled using standard methodology and used to screen for clones expressing previously unknown regulatory proteins, or functional peptides thereof, in cDNA expression libraries made from mRNA from endothelial cells, megakaryocytic cells, or other cells. Of course, any other consecutive sequence of SEQ ID NO. 5 that has been shown to define a regulatory site in the 5′ flanking region of the P-selectin gene could similarly be used in such methods to isolate the particular regulatory protein(s) that bind that sequence using techniques described herein.

IV. Diagnostic Methods

The luciferase expression studies, the gel shift studies, and the lacZ transgene experiments, above, clearly demonstrate that the integrity of the 5′ flanking region of the P-selectin gene is critical for gene expression in endothelial and megakaryocytic cells. A disruption, deletion or other mutation in the sequence of nucleotides in this region can reduce or otherwise alter expression of the P-selectin gene, or heterologous gene properly ligated to this 5′ flanking region. Accordingly, the DNA sequences of the 5′ flanking region of the P-selectin gene can be used as hybridization probes to detect or screen for mutations in the 5′ flanking region of the P-selectin gene of individuals with abnormal levels of expression of P-selectin, particularly those with elevated expression which have clinical symptoms of inflammation. In addition, DNA

molecules including the DNA sequences of the 5′ flanking region of the P-selectin gene can be transcribed to yield the corresponding RNA molecules, if in the judgment of the skilled practitioner it is more desirable to use RNA probes instead of DNA probes.

Calculations and empirical work by Lathe and others, Lathe, *J. Mol. Biol.*, *183*, pages 1-12 (1985); Ikuta et al., *Nucleic Acids Res.*, *15*, pages 797-811 (1987); and Sambrook et al., Molecular Cloning, A Laboratory Manual, Second Edition, pages 11.7, 11.8 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989), incorporated herein by reference, have established helpful guidelines for the skilled practitioner to select the length of probes most sensitive to mismatches in standard hybridization protocols. For example, to detect a single mismatch in a specific DNA sequence of the mammalian genome by hybridization, a probe length of 14 to 20 nucleotides is recommended (see, for example, Sambrook et al., pages 11.7-11.8; Lathe, Table 6, page 10). Accordingly, DNA and RNA molecules including DNA sequences, or the corresponding RNA sequences, which include at least 14 consecutive nucleotides of SEQ ID NO. 5, or functionally equivalent molecules, for example, obtained by hybridization under stringent conditions or substitution of specific bases followed by screening for function, for use as probes in diagnostic hybridization methods to detect mutations in the 5′ flanking region of the P-selectin gene of individuals with altered (for example, abnormally low or high) levels of expression of P-selectin. A whole series of nucleic acid probes including nucleotide sequences consisting essentially of 14 to 20 consecutive nucleotides of SEQ ID NO. 5 can be used to detect

mutations along the entire or a portion of the 5' flanking region of the P-selectin gene.

Standard methods to isolate DNA and RNA from mammalian cells and tissues are well known in the art and can be used to prepare DNA (or RNA) for routine diagnostic screening by hybridization using the probes described herein. See, for examples of methods known to those skilled in the art which can be adapted for use herein using routine variation, Sambrook et al., Molecular Cloning, A Laboratory Manual, Second Edition, pages 6.53-6.54 (DNA); pages 7.6-7.11 (RNA) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989); and Chomczyniski and Sacchi, *Anal. Biochem.*, *162*, pages 156-159 (1987) (RNA), the teachings of which are incorporated herein by reference. Furthermore, methods of carrying out hybridizations (for example, Southern and Northern blot methods) of nucleic acids immobilized on various supports, such as nylon membranes or nitrocellulose filters, using DNA probes described herein labelled with some standard detectable marker, for example, by using commercially available biotinylated, chemiluminescent, fluorescent, enzyme or radioactive labeling systems, are also routine and readily adapted to screening samples of DNA (or RNA) from individuals for alterations in the 5' flanking region of the P-selectin gene. See also, Leary et al., *Proc. Natl. Acad. Sci. USA*, *80*, pages 4045-4049 (1983) (biotinylated probes); and Sambrook et al., Chapter 9 ("Analysis of Genomic DNA by Southern Hybridization"), Chapter 10 ("Preparation of Radiolabeled DNA and RNA Probes")). In addition to using membranes, the advantages of probing nucleic acid samples immobilized to the plastic wells of microtiter plates has recently been advocated, offering

another variation of the available methods of screening DNAs or RNAs by hybridization using nucleic acid probes (see Mitsuashi et al., *Nature*, *357*, pages 519-520 (1992). One of ordinary skill in the art is readily able to select from a variety of standard methods the particular conditions under which hybridizations are carried out in order to diagnostically screen DNA or RNA from individuals for alterations in the 5′ flanking region of the P-selectin gene using the DNA molecules described herein as probes.

V. Therapeutic Methods and Compositions

As the above luciferase expression and the *lacZ* transgene expression studies indicate, nucleic acid molecules containing the 5′ regulatory sequences of the P-selectin gene can be inserted into endothelial or megakaryocytic cells and used to regulate or inhibit heterologous gene expression *in vivo*. In the luciferase experiments, the plasmid pOLUC was used as the vector to carry and express the various recombinant 5′ flanking region-luciferase gene constructs in endothelial cells. However, other vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5′ flanking region-gene construct in endothelial or megakaryocytic cells depending on the preference and judgment of the skilled practitioner (see, for example, Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic acid sequences *in vivo* (see, for example, Mulligan, *Science, 260*, pages 926-932 (1993); United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Recently, a delivery system was developed in which nucleic acid is encapsulated in cationic liposomes which can be

injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, for example, Zhu et al., *Science*, *261*, pages 209-211 (1993); incorporated herein by reference).

The 5′ flanking sequences of the P-selectin gene can also be used to inhibit the expression of the P-selectin gene in cells and thereby affect the inflammatory response. For example, an antisense RNA of all or a portion of the 5′ flanking region of the P-selectin gene can be used to inhibit expression of P-selectin *in vivo*. Expression vectors (for example, retroviral expression vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which is expressed in a cell (see, for example, U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286). Accordingly, DNA containing all or a portion of the sequence of the 5′ flanking region of P-selectin gene can be inserted into an appropriate expression vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the P-selectin gene normally found in the cell. This antisense RNA transcript of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. It is of course necessary to select sequences of the 5′ flanking region that are downstream from the transcriptional start sites for the P-selectin gene to ensure that the antisense RNA contains complementary sequences present on the mRNA. Based on the transcriptional start site analysis (see above and Figure 4) sequences between 4863 and 4842 of SEQ ID NO. 5 are most likely to be transcribed.

Accordingly, the DNA to be inserted into the expression vector for antisense therapy should at least contain these sequences.

Antisense RNA can be generated *in vitro* also, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and viral replication (see, for example, Zamecnik et al., *Proc. Natl. Acad. Sci. USA, 75*, pages 280-284 (1978); Zamecnik et al., *PNAS, 83*, pages 4143-4146 (1986); Wickstrom et al., *Proc. Natl. Acad. Sci. USA, 85*, pages 1028-1032 (1988); Crooke, *FASEB J., 7*, pages 533-539 (1993). Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see, for example, Offensperger et. al., *EMBO J., 12*, pages 1257-1262 (1993) (*in vivo* inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., *Proc. Natl. Acad. Sci. USA, 85*, pages 7079-7083 (1988) (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., *Proc. Natl. Acad. Sci. USA, 85*, pages 7448-7794 (1989) (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., *Nucleic Acids Res, 19*, pages 747-750 (1991) (synthesis of 3′ exonuclease-resistant oligonucleotides containing 3′ terminal

phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5′ flanking region of P-selectin can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, for example, Maher et al., *Science, 245,* pages 725-730 (1989); Orson et al., *Nucl. Acids Res., 19,* pages 3435-3441 (1991); Postal et al., *Proc. Natl. Acad. Sci. USA, 88,* pages 8227-8231 (1991); Cooney et al., *Science, 241,* pages 456-459 (1988); Young et al., *Proc. Natl. Acad. Sci. USA, 88,* pages 10023-10026 (1991); Duval-Valentin et al., *Proc. Natl. Acad. Sci. USA, 89,* pages 504-508 (1992); Blume et al., *Nucl. Acids Res.,* 20, pages 1777-1784 (1992); Grigoriev et al., *J. Biol. Chem., 267,* pages 3389-3395 (1992).

Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, for example, Maher et al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see, for example, Orson et al., (1991); Holt et al., *Mol. Cell. Biol., 8,* pages 963-973 (1988); Wickstrom et al., *Proc. Natl. Acad. Sci. USA, 85,* pages 1028-1032 (1988)). To reduce susceptibility to intracellular degradation, for example, by 3′ exonucleases, a free amine can be introduced to a 3′ terminal

hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (for example, via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992).

Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see, for example, Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., in *Ann. Rev. Biochem., 53*, pages 323-356 (1984) (phosphotriester and phosphite-triester methods); Narang et al., in *Methods Enzymol., 65*, pages 610-620 (1980) (phosphotriester method). Accordingly, DNA sequences of the 5' flanking region of the P-selectin gene described herein can be used to design and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides of SEQ ID NO. 5, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically within the 5' flanking region of a P-selectin gene in order to inhibit expression of the gene in endothelial or megakaryocytic cells.

In addition, Rosenberg et al. (PCT WO 93/01286) teach the topical application of compositions including antisense oligonucleotides, generally 15-30 nucleotides in length, and

Pluronic™ (polypropyl ne oxide - polyethylene oxide block copolymer) gel, which is liquid at 4°C and solid at room temperature. Cells in direct contact with the gel compositions will take up the antisense oligonucleotides which will then base-pair to the complementary mRNA and inhibit expression of the target gene. Other biodegradable polymers can be substituted for the pluronics™ such as the polylactic acid and polyglycolic acid copolymers, polyethylene, and polyorthoesters which can be used to form implants for controlled release of the nucleic acid directly to a tissue where expression is desired.

Compositions including Pluronic™ gel and the antisense oligonucleotides or oligonucleotides complementary to one of the strands of the 5' flanking region (for triplex formation) can be delivered locally to the endothelial cells in blood vessels by using a catheter which is advanced into a vessel and applying the composition directly to the endothelial tissue. Other means of delivering such compositions locally to cells include using infusion pumps (for example, from Alza Corporation, Palo Alto, California) or incorporating the compositions into polymeric implants (see, for example, P. Johnson and J.G. Lloyd-Jones, eds., Drug Delivery Systems (Chichester, England: Ellis Horwood Ltd., 1987), which can effect a sustained release of therapeutic compositions to the immediate area of the implant.

Inhibition of Inflammation by inhibition of P-selectin expression.

The above methods and compositions may be used locally or systemically to inhibit the expression of P-selectin *in vivo* and thereby inhibit inflammation. The ability to inhibit or otherwise regulate the inflammatory response at a site is desirable therapeutically because an inflammatory

response may cause damage to the host if unchecked, since leukocytes release many toxic molecules that can damage normal tissues. These molecules include proteolytic enzymes and free radicals. Examples of pathological situations in which leukocytes can cause tissue damage include injury from ischemia and reperfusion, bacterial sepsis and disseminated intravascular coagulation, adult respiratory distress syndrome, tumor metastasis, and atherosclerosis. Systemic administration of compounds to achieve chronic systemic down-regulation of P-selectin expression may also be useful, for example, in a chronic disorder such as rheumatoid arthritis.

There are a number of other common clinical disorders in which ischemia and reperfusion results in organ injury mediated by adherence of leukocytes to vascular surfaces, including strokes; mesenteric and peripheral vascular disease; organ transplantation; and circulatory shock (in this case many organs might be damaged following restoration of blood flow).

Bacterial sepsis and disseminated intravascular coagulation often exist concurrently in critically ill patients. They are associated with generation of thrombin, cytokines, and other inflammatory mediators, activation of platelets and endothelium, and adherence of leukocytes and aggregation of platelets throughout the vascular system. Leukocyte-dependent organ damage is an important feature of these conditions.

Adult respiratory distress syndrome is a devastating pulmonary disorder occurring in patients with sepsis or following trauma, which is associated with widespread adherence and aggregation of leukocytes in the pulmonary circulation. This leads to extravasation of large

amounts of plasma into the lungs and destruction of lung tissue, both mediated in large part by leukocyt products.

Two related pulmonary disorders that are often fatal are in immunosuppressed patients undergoing allogeneic bone marrow transplantation and in cancer patients suffering from complications that arise from generalized vascular leakage resulting from treatment with interleukin-2 treated LAK cells (lymphokine-activated lymphocytes). LAK cells are known to adhere to vascular walls and release products that are presumably toxic to endothelium. Although the mechanism by which LAK cells adhere to endothelium is not known, such cells could potentially release molecules that activate endothelium and then bind to endothelium by mechanisms similar to those operative in neutrophils.

Tumor cells from many malignancies (including carcinomas, lymphomas, and sarcomas) can metastasize to distant sites through the vasculature. The mechanisms for adhesion of tumor cells to endothelium and their subsequent migration are not well understood, but may be similar to those of leukocytes in at least some cases. The association of platelets with metastasizing tumor cells has been well described, indicating a role for platelets in the spread of some cancers.

Platelet-leukocyte interactions are believed to be important in atherosclerosis. Platelets might have a role in recruitment of monocytes into atherosclerotic plaques; the accumulation of monocytes is known to be one of the earliest detectable events during atherogenesis. Rupture of a fully developed plaque may not only lead to platelet deposition and activation and the

promotion of thrombus formation, but also the early recruitment of neutrophils to an area of ischemia.

Modifications and variations of the present invention, will be recognized by those skilled in the art from the foregoing detailed description. It is understood that such modifications and variations are intended to come within the scope of this invention as defined in the specification, and that this invention is not limited by the specific embodiments that have been presented by way of example.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Board of Regents of the University of Oklahoma
(ii) TITLE OF INVENTION: Expression Control Sequences of the P-Selectin Gene
(iii) NUMBER OF SEQUENCES: 17
(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Atlanta
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(F) ZIP: 30309-4530
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (404)-815-6508
(B) TELEFAX: (404)-815-6555

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: synthetic DNA
(iii) HYPOTHETICAL: NO
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(x) PUBLICATION INFORMATION:
(A) AUTHORS: Johnston, G. I.
Cook, R. G.
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(C) JOURNAL: Abstract 1238 Supplement II Circulation
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(F) PAGE: II-310
(G) DATE: October-1988
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear
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ATGACACCAA CTGTAGCTTC CGCTGTGCTG AAGGTTTCAT GCTGAGAGGA GCCGATATAG    1320
TTCGGTGTGA TAACTTGGGA CAGTGGACAG CACCAGCCCC AGTCTGTCAA GCTTTGCAGT    1380
GCCAGGATCT CCCAGTTCCA AATGAGGCCC GGGTGAACTG CTCCCACCCC TTCGGTGCCT    1440
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TTAGGTACCA GTCAGTCTGC AGCTTCACCT GCAATGAAGG CTTGCTCCTG GTGGGAGCAA   1500
GTGTGCTACA GTGCTTGGCT ACTGGAAACT GGAATTCTGT TCCTCCAGAA TGCCAAGCCA   1560
TTCCCTGCAC ACCTTTGCTA AGCCCTCAGA ATGGAACAAT GACCTGTGTT CAACCTCTTG   1620
GAAGTTCCAG TTATAAATCC ACATGTCAAT TCATCTGTGA CGAGGGATAT TCTTTGTCTG   1680
GACCAGAAAG ATTGGATTGT ACTCGATCGG GACGCTGGAC AGACTCCCCA CCAATGTGTG   1740
AAGCCATCAA GTGCCCAGAA CTCTTTGCCC CAGAGCAGGG CAGCCTGGAT TGTTCTGACA   1800
CTCGTGGAGA ATTCAATGTT GGCTCCACCT GTCATTTCTC TTGTAACAAT GGCTTTAAGC   1860
TGGAGGGGCC CAATAATGTG GAATGCACAA CTTCTGGAAG ATGGTCAGCT ACTCCACCAA   1920
CCTGCAAAGG CATAGCATCA CTTCCTACTC CAGGGTTGCA ATGTCCAGCC CTCACCACTC   1980
CTGGGCAGGG AACCATGTAC TGTAGGCATC ATCCGGGAAC CTTTGGTTTT AATACCACTT   2040
GTTACTTTGG CTGCAACGCT GGATTCACAC TCATAGGAGA CAGCACTCTC AGCTGCAGAC   2100
CTTCAGGACA ATGGACAGCA GTAACTCCAG CATGCAGAGC TGTGAAATGC TCAGAACTAC   2160
ATGTTAATAA GCCAATAGCG ATGAACTGCT CCAACCTCTG GGGAAACTTC AGTTATGGAT   2220
CAATCTGCTC TTTCCATTGT CTAGAGGGCC AGTTACTTAA TGGCTCTGCA CAAACAGCAT   2280
GCCAAGAGAA TGGCCACTGG TCAACTACCG TGCCAACCTG CCAAGCAGGA CCATTGACTA   2340
TCCAGGAAGC CCTGACTTAC TTTGGTGGAG CGGTGGCTTC TACAATAGGT CTGATAATGG   2400
GTGGGACGCT CCTGGCTTTG CTAAGAAAGC GTTTCAGACA AAAAGATGAT GGGAAATGCC   2460
CCTTGAATCC TCACAGCCAC CTAGGAACAT ATGGAGTTTT TACAAACGCT GCATTTGACC   2520
CGAGTCCTTA AGGTTTCCAT AAACACCCAT GAATCAAAGA CATGGAATTA CCTTAGATTA   2580
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GCTCTGGACC AGCCTGTTGG ACCCGCTCTG GACCAACCCT GTTTCCTGAG TTTGGGATTG    2640

TGGTACAATC TCAAATTCTC AACCTACCAC CCCTTCCTGT CCCACCTCTT CTCTTCCTGT    2700

AACACAAGCC ACAGAAGCCA GGAGCAAATG TTTCTGCAGT AGTCTCTGTG CTTTGACTCA    2760

CCTGTTACTT GAAATACCAG TGAACCAAAG AGACTGGAGC ATCTGACTCA CAAGAAGACC    2820

AGACTGTGGA GAAATAAAAA TACCTCTTTA TTTTTTGATT GAAGGAAGGT TTTCTCCACT    2880

TTGTTGGAAA GCAGGTGGCA TCTCTAATTG GAAGAAATTC CTGTAGCATC TTCTGGAGTC    2940

TCCAGTGGTT GCTGTTGATG AGGCCTCTTG GACCTCTGCT CTGAGGCTTC CAGAGAGTCC    3000

TCTGGATGGC ACCAGAGGCT GCAGAAGGCC AAGAATCAAG CTAGAAGGCC ACATGTCACC    3060

GTGGACCTTC CTGCCACCAG TCACTGTCCC TCAAATGACC CAAAGACCAA TATTCAAATG    3120

CGTAATTAAA AGAATTTTCC CC                                              3142
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 830 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Asn Cys Gln Ile Ala Ile Leu Tyr Gln Arg Phe Gln Arg Val
1               5                  10                  15

Val Phe Gly Ile Ser Gln Leu Leu Cys Phe Ser Ala Leu Ile Ser Glu
            20                  25                  30

Leu Thr Asn Gln Lys Glu Val Ala Ala Trp Thr Tyr His Tyr Ser Thr
        35                  40                  45
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Lys Ala Tyr Ser Trp Asn Ile Ser Arg Lys Tyr Cys Gln Asn Arg Tyr
    50                  55                  60

Thr Asp Leu Val Ala Ile Gln Asn Lys Asn Glu Ile Asp Tyr Leu Asn
65                  70                  75                  80

Lys Val Leu Pro Tyr Tyr Ser Ser Tyr Tyr Trp Ile Gly Ile Arg Lys
                85                  90                  95

Asn Asn Lys Thr Trp Thr Trp Val Gly Thr Lys Lys Ala Leu Thr Asn
            100                 105                 110

Glu Ala Glu Asn Trp Ala Asp Asn Glu Pro Asn Asn Lys Arg Asn Asn
        115                 120                 125

Glu Asp Cys Val Glu Ile Tyr Ile Lys Ser Pro Ser Ala Pro Gly Lys
    130                 135                 140

Trp Asn Asp Glu His Cys Leu Lys Lys Lys His Ala Leu Cys Tyr Thr
145                 150                 155                 160

Ala Ser Cys Gln Asp Met Ser Cys Ser Lys Gln Gly Glu Cys Leu Glu
                165                 170                 175

Thr Ile Gly Asn Tyr Thr Cys Ser Cys Tyr Pro Gly Phe Tyr Gly Pro
            180                 185                 190

Glu Cys Glu Tyr Val Arg Glu Cys Gly Glu Leu Glu Leu Pro Gln His
        195                 200                 205

Val Leu Met Asn Cys Ser His Pro Leu Gly Asn Phe Ser Phe Asn Ser
    210                 215                 220

Gln Cys Ser Phe His Cys Thr Asp Gly Tyr Gln Val Asn Gly Pro Ser
225                 230                 235                 240
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Lys Leu Glu Cys Leu Ala Ser Gly Ile Trp Thr Asn Lys Pro Pro Gln
                245                 250                 255

Cys Leu Ala Ala Gln Cys Pro Pro Leu Lys Ile Pro Glu Arg Gly Asn
            260                 265                 270

Met Ile Cys Leu His Ser Ala Lys Ala Phe Gln His Gln Ser Ser Cys
        275                 280                 285

Ser Phe Ser Cys Glu Glu Gly Phe Ala Leu Val Gly Pro Glu Val Val
    290                 295                 300

Gln Cys Thr Ala Ser Gly Val Trp Thr Ala Pro Ala Pro Val Cys Lys
305                 310                 315                 320

Ala Val Gln Cys Gln His Leu Glu Ala Pro Ser Glu Gly Thr Met Asp
                325                 330                 335

Cys Val His Pro Leu Thr Ala Phe Ala Tyr Gly Ser Ser Cys Lys Phe
            340                 345                 350

Glu Cys Gln Pro Gly Tyr Arg Val Arg Gly Leu Asp Met Leu Arg Cys
        355                 360                 365

Ile Asp Ser Gly His Trp Ser Ala Pro Leu Pro Thr Cys Glu Ala Ile
    370                 375                 380

Ser Cys Glu Pro Leu Glu Ser Pro Val His Gly Ser Met Asp Cys Ser
385                 390                 395                 400

Pro Ser Leu Arg Ala Phe Gln Tyr Asp Thr Asn Cys Ser Phe Arg Cys
                405                 410                 415

Ala Glu Gly Phe Met Leu Arg Gly Ala Asp Ile Val Arg Cys Asp Asn
            420                 425                 430
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Leu Gly Gln Trp Thr Ala Pro Ala Pro Val Cys Gln Ala Leu Gln Cys
435 440 445

Gln Asp Leu Pro Val Pro Asn Glu Ala Arg Val Asn Cys Ser His Pro
450 455 460

Phe Gly Ala Phe Arg Tyr Gln Ser Val Cys Ser Phe Thr Cys Asn Glu
465 470 475 480

Gly Leu Leu Leu Val Gly Ala Ser Val Leu Gln Cys Leu Ala Thr Gly
485 490 495

Asn Trp Asn Ser Val Pro Pro Glu Cys Gln Ala Ile Pro Cys Thr Pro
500 505 510

Leu Leu Ser Pro Gln Asn Gly Thr Met Thr Cys Val Gln Pro Leu Gly
515 520 525

Ser Ser Ser Tyr Lys Ser Thr Cys Gln Phe Ile Cys Asp Glu Gly Tyr
530 535 540

Ser Leu Ser Gly Pro Glu Arg Leu Asp Cys Thr Arg Ser Gly Arg Trp
545 550 555 560

Thr Asp Ser Pro Pro Met Cys Glu Ala Ile Lys Cys Pro Glu Leu Phe
565 570 575

Ala Pro Glu Gln Gly Ser Leu Asp Cys Ser Asp Thr Arg Gly Glu Phe
580 585 590

Asn Val Gly Ser Thr Cys His Phe Ser Cys Asn Asn Gly Phe Lys Leu
595 600 605

Glu Gly Pro Asn Asn Val Glu Cys Thr Thr Ser Gly Arg Trp Ser Ala
610 615 620

Thr Pro Pro Thr Cys Lys Gly Ile Ala Ser Leu Pro Thr Pro Gly Leu
625 630 635 640

Gln Cys Pro Ala Leu Thr Thr Pro Gly Gln Gly Thr Met Tyr Cys Arg
645 650 655

His His Pro Gly Thr Phe Gly Phe Asn Thr Thr Cys Tyr Phe Gly Cys
660 665 670

Asn Ala Gly Phe Thr Leu Ile Gly Asp Ser Thr Leu Ser Cys Arg Pro
675 680 685

Ser Gly Gln Trp Thr Ala Val Thr Pro Ala Cys Arg Ala Val Lys Cys
690 695 700

Ser Glu Leu His Val Asn Lys Pro Ile Ala Met Asn Cys Ser Asn Leu
705 710 715 720

Trp Gly Asn Phe Ser Tyr Gly Ser Ile Cys Ser Phe His Cys Leu Glu
725 730 735

Gly Gln Leu Leu Asn Gly Ser Ala Gln Thr Ala Cys Gln Glu Asn Gly
740 745 750

His Trp Ser Thr Thr Val Pro Thr Cys Gln Ala Gly Pro Leu Thr Ile
755 760 765

Gln Glu Ala Leu Thr Tyr Phe Gly Gly Ala Val Ala Ser Thr Ile Gly
770 775 780

Leu Ile Met Gly Gly Thr Leu Leu Ala Leu Leu Arg Lys Arg Phe Arg
785 790 795 800

Gln Lys Asp Asp Gly Lys Cys Pro Leu Asn Pro His Ser His Leu Gly
805 810 815

Thr Tyr Gly Val Phe Thr Asn Ala Ala Phe Asp Pro Ser Pro
820 825 830

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4866 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGCTTCCTG TACCTGGAAT ATTAATATTT TATTTCAGAT TTGGGAAATT TTCAGCTAGC 60

AATCTTTAAA TATGCTTTCT GACCCCCTTT TCCTCTATTT TCTCCTTCTT AAACTACTGT 120

AATGTGAACA TTAGCTCTCT TTTTTAATTT TTAATTTAAT TTTTGTTTTT ATTTTTTGAG 180

ATGCAGTCTC ACTCTGTCAC CCAGGCTGGA GTGCAGTGGC ATGATCTCAG CTCACTGCAA 240

CCTTTGCCTT CTAGGTTGAA GAGATTCTGC TGCCTCAGTC TCCCCATGAG CTGGGATTAC 300

AGCATGTGCC ACAATCCCTG GCTAATTTTT TTGTATTTTT AGTAGAGACT GGGTTTCACC 360

ATGTTGGTCA GGCTGGTCTT GACTCCTGAC CTCAGGTGAT CCACTCACCT TGGCCTCCCA 420

AAGCGCTGGG ATTATGGCAT GAGCCACTGA GTCTGGCTGA ATGTTAGCTC TCTTGATGCT 480

GTCCCATAAA TCTTGTAGGC TTTCATCATT TCTTTTCATT CTTTTTTCTC CTCTCACTGT 540

ATATTTTCAA AAACCTGTCT TCAGTTCACA GATTCTTTCT TCTGCTTGAT CAAGTCTGCT 600

ACTGGTGATT TCTACTGCAT TTCTCACTTC ATTCATTATA TTTTTCAGCT CCAATTTCTT 660

TTATGATTTC AATCTTTCTG TTACATTTCT TATGTTGTGC ATTTATTGTT TCTCTGATTT 720

CACCAAATTG TTTCTCTGTG TTTGCTTCAA AGTAACTGAG CTTCTTTAAA AACAATTATC 780

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TTGAATCCAT TGTCAGGCCA TTTGTAGTAC TCCATTTCTT TTGGGTCAGC TACTGGGAAA     840
TTATTGTGTT TCTTAGGTGG TGATATTTTA ATTTGGGTTT TCATGTTTCT TGCTGCCTTA     900
CACTGCTGTC TGAGCATCTG GTGGATCTGC CCCAATTTCA GGCTGTATGG GCTGACTTTG     960
GTGGAGAAAT ACCTTCTTAT GTGGAATAAT GCGAGGATGC TGGCTGGGTG GGATGCAAAA    1020
GTTCTGACTT CAGTAGGGGC AAAGCTGTGT GGTCTCCATG CAGATCTGTC AGCTGAGGTT    1080
GGTGTTAGTG AATACTACAG GGATCCTTAG AGGCCAACAC TGTGGGTATC TACAGTGGCA    1140
ATGAGGCTGT TGAGGTTTTC AATTGTGACA AGTCCTCCAT ATCTCTTTTT TTCCCCACCT    1200
GGGAAGTCAT GACTGAGGAC ATCCCTCTTG GAATTAGGTC TAACTTGCAG GCCTGCTCCT    1260
GGTGGTGGTG ACACTGGTGT CTGATGAACA GTGCCCATGG AGTGGCCAAG AGCCAAGGCC    1320
TGAAGCATGG GCATGCATGG AGGGACCACA GCACCAGATT CAATTGTAGC AATGGTACCA    1380
GTGCCCAAGG CACAGGCATA CTTACTATCA CATTGATAAT GGTGTGTAAA ATGCAGGTAC    1440
TTATAAAGCA GCTAAGGAGC CAGGGACTTT ACTGCATGCA TACGCAGAGC TACAGTGGCT    1500
CCAGGATCCA GGGTGTGGGC TAGCTCTCCT TGGTGGCTGA GCTGGTGACT AGAGCATGGA    1560
CAGGCACAGA GAAACCTTGA CTCTAGGACC CAGGGTGTTC ACTAGCTCAC TATAGTGGTG    1620
GCTCTGGTGT TGGAGGTGTG GGTGTGTGTA GTACAGCCTC AGAGACAGGG TCTGGAGCGC    1680
AGGTGTGCAC ATTACTACAG CAGCTCTGGA GTTGAGAATA TGGGTTACCT TTCTACAGTG    1740
GCTGAACTAG TGTCTGGAGC AAAGACTTTC ACAGAGAGAA CTTGGCTTGG GGTCCCAGGG    1800
TGAGATCTAG TTCACAACAG CAGTGACTCC AGTGTCTGAG ACATGAGGAG GTGCACTGCA    1860
GCCACAGAGC CACAGTCCAG AGTGTGAATA TCTGTAGAGC AGCCACAACT TTTGGGGATC    1920
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AGGAACACAC ATAGAATTGT GAGAGGTGGT AACCCTGGCC CCAGTCCTGG CGAGTGCAAC   1980
AATAGCTGCT TCTTGGTGAG GGGGTGTGAG GGGTAGTGCA ACTGTGTTTC CCTTTTTAGC   2040
ATCCTGCTAT GGGAATGGCT GTTGGACAAA AGATGCCAGT GTCCTCTGTG GAGCAGGACA   2100
CTGGGGGCCT CAGTGGCTCT GTGTCACATG ACTGACACAG ATAGCCTACA AATTTCTTTA   2160
TTCGTAGCTA TCTCCTGGTG TCTCATATAT GCCAGTCTCA CCGGTGATTC TTCTACATGA   2220
ATATTCTTTC TTTTCTCCAT TGTGTTGTTC CAAATTCTTT AACAGGCTCT TGAGCCCCAT   2280
CCCCCAACTC CCCACCCTTG TGAGGGCTAT TTTGGTTTGT GTATAACTGT CTATGTTTGT   2340
TTTTTTGTTG GGGCATAAGG CTGACATCTC CTACTCCACC ATCTTGCTAA TGTCACCTGC   2400
ATAGGAATCT TTTTATGCTT TCCTTATATT CACTAAAATT TAACAATATC AAACTTAAAA   2460
ACATATGATC AATTGAACTT ATTAATATCA AACTTATTAT AAATAAGAAA CTACCAGGCT   2520
GGGCATGGTG GCTCATGCCT GTAATCCCAA CATTTTGGGA GGCTGAGGTG AAAGGATCAC   2580
TTGAGCCCAG GAATTCAAGA CCAGCCTGGG AAATATAGAG AGACCCTATC TCTAGAGATT   2640
TTTTTTTTTA ATTAGCCAGT AGTGATGGCA CACATCTATA GTCCCAGCTA CTCAGGAGGC   2700
TGAGGTGGGA GAATTGCTTG AGCCCAGGAG GTCAAGGCTG CAGCAAGCAG TAATCATGCC   2760
ACTGCACTCC AGCCTGGGCC GCAGAGTGAG ACCCTGTCTC AAAAAAAGAA CCTACTAGTC   2820
TACATACCAC ACTTCTTCAT CCCCATCTGA GACTATATAT ATTTTTTCTA ACATGAGGCA   2880
ATGCCAAAAA GAGGAGCTGG TGAGTGAAAG TAAGAACAGA AAGACATGGA GGCAAGTCTT   2940
ATAGAATAAT AGCCAACACT TAAACTTACA CTTAACAGCG TGATAGGTAT TGTTCCAAAC   3000
ACATTAAATT CATTTAATGG TCCTTACATG TCTATGTATT TGGTGATTAT TATCCTTATT   3060
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ATTCACATTG CTGAGTGTAT TATTCTGTTC TCATGATGCT GATAGAGACA TACCCGAGAC 3120

TGGATAACTT ATTAAAAAAA AAAAGGTTTA ATGGACTCAC AGTTCCACGT GGATGGGGAG 3180

TCCTCACAAT CATGGTAGAA AGCAAAAGAC ACGTCTTACA TGGCAGCAGG GAAGAGAGAG 3240

AAATGAGAAC CAAACAAAAG GGGTTTCCCC TTATAAAACC ATCAGCTCTC ATGCGACTTA 3300

TTCACTACCA TGAGAACAGT ATGGGGGAAA CCACCCCCAT GATTCAATGA TCTACCAGGT 3360

GCCTCCCACA ACCTGTGGGA ATTATGGGAG CTACAATTCC AGATGAGATT TGGGTGGGGA 3420

CACAGCCAAA CCACATCACT GAGGAAACTG AGTTATAGGG AGATTAGTAA CGCCCAACAC 3480

AGCTGGTAGG TGGTGGAGCC AGGCAGTCTG ACTCTAGGGT CTGGACTCTG AACTGCATCA 3540

TGCTGCCAAG AAGTTCCTCA TTTTTTCCTC TCTCTAAGTT TCCCTTATTC CCCTACAGTC 3600

ATTCCTTCAA CAGCATTTCC TTCACCATCT TTTCTACTTC TACTATATAA TTAATTTTTT 3660

CTTCTTGGTC CCAAATTCCA ACGTGCAAAT GCAGCCTTAT ATACCCTAAT TCATCTTTAC 3720

CTTTAGACTT TCTTCCAATG TTTCTACTTC ATTCCATTTT AAATTTATCC ATGAGATGCC 3780

TATTTACAAG CTGTAACCAT CATGAAGTGA ATGAAGAATA ATACCTACTA CTGTACAATA 3840

GAATTCCAAG AGTATAAATA GGAGTTATGG CTTTCTGACT TGAAACTAAA TACTTGATAC 3900

TTGATTTTGC TGTCTGAGAT CAATCTGAAA AGTAATAATA ATCACTAACA TTTGTTGAGC 3960

ATCAATTGTG GGCCAAGTGT CATTTCAATC ACTCTGTACA TATTAACTCA TTTCATCCTA 4020

CAACAACCCG GTGAGGCAAG TTCTGTTATT CTGTTTTACA GTTGAGGAAA CAGAGGCATA 4080

GAGAGCTTAA GTAGTTTGCC CAGTAGATAG CCAGAAGAGG AGCCAGGATG GGTCTCGGGC 4140

AGTTTAACAG CACAGCTGAA GTCTTAACCA CTATGCCAAC AGCTTTTTGG TCCTACACAT 4200

CCCATGGGAA GAGGAAAATA AAAAGGTATC TATTTGTATA CCTTTTTATT TCTGATATAA 4260

GAAGCAGAAT TCCTTTCACA TGACCTATGT CTATTTAATA CGTCATTTTG AAACTTACCA 4320

ATAAAATTTC CCAAGCGCCA GAAAACTGTT AGTGGCTTTT TCCATTCTTC TCTATTTTTT 4380

TTTGTGCTAC TAATTTTGCT TCTTTCCCTC AGAAGGCTGC CGGAATAGTA AACATTCACT 4440

GACATGTCAT AATTACTGGA AAATGGGCAC TGGAAAATCA CATTGTAATT AATTCAAAGC 4500

ATGTTTTCCA AATGTACTAC TTTAAATTGG AGCTTATATC ATAATCCAAG GAAACCTTTG 4560

TGTGTGTACT GTTCCCACAT TGCTCAGCCT GGGATATCCA GGAGTAATTC ACCTTGCGCC 4620

TGCCTCCAGA CCATCTTCCA TGGAAGGGGG TGACCCCTTG CCTCTTGGCA ACCACTATTT 4680

CTAAGCTGCC AACATTACTC TTGCATTATC AACATTCTAA CTTCATGGGA AGGGCTGTGG 4740

TGAGTTTCTG GAATGTGAAT AGGAAGTTGT TTTTCTAAAC AGCCTGACAC TGAGGGGAGG 4800

CAGTGAGACT GTAAGCAGTC TGGGTTGGGC AGAAGGCAGA AAACCAGCAG AGTCACAGAG 4860

GAGATG 4866

(2) INFORMATION FOR SEQ ID NO:6:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 27 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: cDNA
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTCTGGTTTG TTAGTTCAGA GATCAGG 27

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATGTATATC TCCACGCAGT CCTCG 25

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAATTCGAGC TCGGTACCTT TTTTTTTTT TTTTT 35

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCGAGC TCGGTACC 18

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTCGACTCTA GAATCAGCCC AGTTCTCAGC 30

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGCTGGGGA TTCCCCATCT 20

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTGGTTCCA AG

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTGGTTTTA AG 12

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTCCATCCA AG 12

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCGAGCAGGA AGTGACGTCG A 21

(2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCCAAACCG GAAGCATGTG 20

(2) INFORMATION FOR SEQ ID NO:17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTTGCGGA ACGGAAGCGG AAACCGCCGG ATCG 34

We claim:

1. A method for the regulated expression of a gene in endothelial cells or megakaryocytes comprising fusing the 5′ end of the coding sequence of the gene to the 3′ end of a DNA molecule comprising a nucleotide sequence consisting essentially of nucleotides 1 to 4863 of SEQ ID NO. 5, or portions thereof effective to control expression of a gene, to yield a recombinant gene construct; inserting the recombinant gene construct into endothelial cells or megakaryocytes; and assaying for expression of the recombinant gene inserted into the endothelial cells or megakaryocytes.

2. The method of claim 1 for the regulated expression of a gene encoding a protein wherein the 5′ end of the coding sequence of the gene is fused to the 3′ end of a DNA molecule comprising a nucleotide sequence of at least eight contiguous nucleotides of a sequence selected from the group consisting of nucleotides 1 to 4863, 1 to 4851, 3525 to 4851, 3525 to 4863, 3732 to 4866, 4163 to 4851, 4163 to 4863, 4206 to 4866, 4212 to 4866, 4405 to 4851, 4405 to 4863, 4446 to 4469, 4453 to 4461, 4457 to 4866, 4471 to 4866, 4505 to 4866, 4555 to 4666, 4555 to 4851, 4555 to 4863, 4615 to 4666, 4615 to 4764, 4615 to 4851, 4615 to 4861, 4615 to 4863, 4626 to 4650, 4632 to 4672, 4635 to 4646, 4636 to 4645, 4641 to 4866, 4642 to 4664, 4645 to 4866, 4646 to 4650, 4646 to 4657, 4650 to 4669, 4667 to 4863, 4667 to 4717, 4717 to 4863, 4722 to 4731, 4727 to 4866, 4747 to 4757, 4734 to 4758, 4736 to 4764, 4736 to 4863, 4745 to 4753, 4761 to 4866 of SEQ ID NO. 5, and combinations thereof.

3. The method of claim 1 wherein the gene encodes P-selectin.

4. A method for screening for defects in the 5' flanking region of the P-selectin gene of individuals comprising hybridizing nucleic acid from cells of the individuals to a nucleic acid probe molecule comprising a nucleotide sequence consisting essentially of nucleotides 1 to 4863 of SEQ ID NO. 5, or portions thereof consisting essentially of at least fourteen consecutive nucleotide bases.

5. The method of claim 4 wherein the cells are selected from the group consisting of megakaryocytes and endothelial cells.

6. The method of claim 4 wherein the probe molecule comprises a nucleotide sequence consisting essentially of sequences selected from the group consisting of any one of the sequences defined by nucleotides 1 to 4851, 3525 to 4851, 3525 to 4863, 3732 to 4866, 4163 to 4851, 4163 to 4863, 4206 to 4866, 4212 to 4866, 4405 to 4851, 4405 to 4863, 4446 to 4469, 4453 to 4461, 4457 to 4866, 4471 to 4866, 4505 to 4866, 4555 to 4666, 4555 to 4851, 4555 to 4863, 4615 to 4666, 4615 to 4764, 4615 to 4851, 4615 to 4861, 4615 to 4863, 4626 to 4650, 4632 to 4672, 4635 to 4646, 4636 to 4645, 4641 to 4866, 4642 to 4664, 4645 to 4866, 4646 to 4650, 4646 to 4657, 4650 to 4669, 4667 to 4863, 4667 to 4717, 4717 to 4863, 4722 to 4731, 4727 to 4866, 4747 to 4757, 4734 to 4758, 4736 to 4764, 4736 to 4863, 4745 to 4753, 4761 to 4866 of SEQ ID NO. 5, and combinations thereof.

7. An isolated nucleic acid molecule comprising a nucleotide sequence consisting essentially of nucleotides 1 to 4863 of SEQ ID NO. 5, or portions thereof consisting essentially of at least fourteen consecutive nucleotides of SEQ ID NO. 5.

8. The isolated nucleic acid molecule of claim 7 comprising a nucleotide sequence consisting essentially of the consecutive nucleotides of SEQ ID NO. 5 selected from the group consisting of nucleotides 1 to 4851, 3525 to 4851, 3525 to 4863, 3732 to 4866, 4163 to 4851, 4163 to 4863, 4206 to 4866, 4212 to 4866, 4405 to 4851, 4405 to 4863, 4446 to 4469, 4453 to 4461, 4457 to 4866, 4471 to 4866, 4505 to 4866, 4555 to 4666, 4555 to 4851, 4555 to 4863, 4615 to 4666, 4615 to 4764, 4615 to 4851, 4615 to 4861, 4615 to 4863, 4626 to 4650, 4632 to 4672, 4635 to 4646, 4636 to 4645, 4641 to 4866, 4642 to 4664, 4645 to 4866, 4646 to 4650, 4646 to 4657, 4650 to 4669, 4667 to 4863, 4667 to 4717, 4717 to 4863, 4722 to 4731, 4727 to 4866, 4747 to 4757, 4734 to 4758, 4736 to 4764, 4736 to 4863, 4745 to 4753, 4761 to 4866 of SEQ ID NO. 5, and combinations thereof.

9. The isolated nucleic acid molecule of claim 7 further comprising a gene, wherein the 5′ end of the gene is ligated to the 3′ end of the nucleic acid molecule.

10. The isolated nucleic acid molecule of claim 9 inserted into cells capable of expressing the ligated gene.

11. The isolated nucleic acid molecule of claim 10 wherein the ligated gene is inserted into the cells in an expression vehicle capable of replicating in the cells.

12. The isolated nucleic acid molecule of claim 10 wherein the ligated gene is stably incorporated into the genome of the cells.

13. The isolated nucleic acid molecule of claim 12 wherein the cells are transgenic animals.

14. The isolated nucleic acid molecule of claim 7 inserted into an expression vector.

15. The isolated nucleic acid molecule of claim 14 wherein the vector is a retroviral vector.

16. The isolated nucleic acid molecule of claim 7 further comprising a pharmaceutically acceptable carrier for administration to a person in need of treatment thereof.

17. The isolated nucleic acid molecule of claim 16 wherein the carrier is a polymeric material providing controlled release of the nucleic acid molecule.

18. The isolated nucleic acid molecule of claim 7 wherein an intercalating agent is covalently linked to a 5′ terminal phosphate.

19. The isolated nucleic acid molecule of claim 18 wherein the intercalating agent is an acridine.

20. The isolated nucleic acid molecule of claim 7 wherein an amine is covalently linked to a 3′ terminal hydroxyl group.

21. The isolated nucleic acid molecule of claim 7 wherein cytosine residues are methylated.

22. A method of inhibiting P-selectin expression in cells expressing P-selectin comprising inserting into the cells a nucleic acid molecule comprising a nucleotide sequence consisting essentially of at least fifteen consecutive nucleotides of SEQ ID NO. 5.

23. The method of claim 22 for the regulated expression of a gene encoding a protein wherein the 5′ end of the coding sequence of the gene is fused to the 3′ end of a DNA molecule comprising a nucleotide sequence consisting essentially of sequences selected from the group consisting of sequences between nucleotide 4769 and nucleotide 1 of SEQ ID No. 5.

24. The method of claim 22 comprising inserting into the cells a nucleotide sequence forming a triplex structure with any of the sequence between nucleotides 4864 and 1 of SEQ ID No. 5.

25. A method for isolating a gene transcriptional regulatory protein, or functional DNA-binding peptide portion thereof, comprising binding to the regulatory protein or peptide portion, an isolated DNA molecule comprising a nucleotide sequence of at least eight contiguous nucleotides of SEQ ID NO. 5.

26. The method of claim 25 for isolating a gene transcriptional regulatory protein, or functional DNA-binding peptide portion thereof, comprising binding to the regulatory protein or peptide portion, an isolated DNA molecule comprising a nucleotide sequence consisting essentially of sequences selected from the group consisting of nucleotides 1 to 4863, 1 to 4851, 3525 to 4851, 3525 to 4863, 3732 to 4866, 4163 to 4851, 4163 to 4863, 4206 to 4866, 4212 to 4866, 4405 to 4851, 4405 to 4863, 4446 to 4469, 4453 to 4461, 4457 to 4866, 4471 to 4866, 4505 to 4866, 4555 to 4666, 4555 to 4851, 4555 to 4863, 4615 to 4666, 4615 to 4764, 4615 to 4851, 4615 to 4861, 4615 to 4863, 4626 to 4650, 4632 to 4672, 4635 to 4646, 4636 to 4645, 4641 to 4866, 4642 to 4664, 4645 to 4866, 4646 to 4650, 4646 to 4657, 4650 to 4669, 4667 to 4863, 4667 to 4717, 4717 to 4863, 4722 to 4731, 4727 to 4866, 4747 to 4757, 4734 to 4758, 4736 to 4764, 4736 to 4863, 4745 to 4753, 4761 to 4866 of SEQ ID NO. 5, and combinations thereof.